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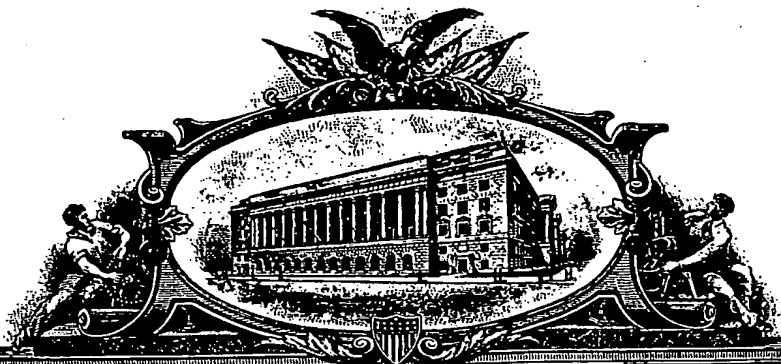Filing date: 15 October 2003 (15.10.2003)

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INVENTOR(S)

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Additional inventors are being named on the __0__ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

COLORECTAL ANTIGEN

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OR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

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COLORECTAL ANTIGEN

The present invention relates to a novel, diagnostic antigen for colorectal cancer, uses thereof, and especially the use thereof in immunotherapeutic treatments for colorectal cancer.

Colon cancer is a leading cause of mortality in Western countries. Despite the improvement of surgery and chemotherapy treatments, the five-year survival rate has not significantly altered over several decades (1, 2). Immunological therapies have been intensively investigated in patients with melanoma, where treatment with IL-2, as well as the adoptive transfer of in-vitro cultured tumour infiltrating lymphocytes (TIL), has been found to result in cancer regression in a significant percentage of patients (3, 4).

In contrast, immunotherapy has not provided a benefit to colorectal cancer patients, which may be due to the poor immunological characterization of this cancer, limiting the treatment options for patients with this disease (5, 6). The presence of a $CD8^+$ T cell infiltrate in colon cancer has prognostic value (7); nevertheless, the presence of an inflammatory infiltrate was not linked to systemic immunity against cancer in this report. The loss of HLA class I expression both *in vitro* and *in vivo* has frequently been described in colorectal cancers, and appears to be associated with tumour progression (8-10).

The limited availability of in-vitro established tumour lines and specific T lymphocytes has in addition hindered analysis of the role of the immune system in colorectal cancer. Although a large number of tumour associated antigens (TAA) have been identified, the majority of these are either limited in their expression to melanoma or are expressed in melanoma as well as in a number of other histologies, including breast, ovarian, lung and prostate tumours (11).

Candidate antigens that appear to be over-expressed in colon cancer such as carcinoembryonic antigen (CEA), the epithelial cell adhesion molecule EP-CAM, HER-

2

2/neu and cyclophilin B, have been evaluated as potential targets for colorectal cancer therapy by carrying out *in vitro* sensitizations of PBMC with candidate peptides from these molecules that bind to particular HLA alleles.

However, a relatively small number of potential epitopes have been identified using this approach and the T cells that have been generated, using many of these peptides, did not efficiently recognise native, unmanipulated tumour cells (12-15).

We have now identified a new tumour associated antigen for colorectal cancer that is capable of eliciting a T cell-mediated immune response.

Thus, in a first aspect, the present invention provides a method for stimulating immunity against colorectal cancer, comprising stimulating the production of antibodies against the human homologue of the Socius gene product, wherein the alanine residue at position 399 is substituted by a valine residue. The coding sequence and the transcript thereof for the homologue are preferably as shown in Figure 5, which shows the relationship of genetic sequence with the COA-1 transcript.

What is particularly surprising is that it has been established that an epitope located between amino acids 371 and 384, inclusive, of the COA-1 transcript is responsible for stimulating immunity against the tumour variant of the protein, and that it is not necessary for the immunising peptide to comprise the mutation at position 399.

The immunising peptide comprises an epitopic portion of the peptide TLYQDDTLTLQAAG. This sequence may be supplemented with additional sequences at either end, up to and including the entire remaining sequences of COA-1, and even additional sequences beyond that, if desired, such as might be encountered with a fusion protein, for example. As demonstrated herein, more specific supplemental sequences, including FSTFPP at the N-terminus and/or LVPKAA at the C-terminus both permit stimulation. It will be appreciated that, in general, an epitope need not be as long as 14 amino acids, and that a deletion of a few amino acid residues from either end of the epitope should still serve to produce immunity.

It has also been established that the epitope is preferentially expressed by antigen presenting cells in association with the alleles HLA DRβ1*0402 or HLA

DRβ1*1301. It will be appreciated that these sympathetic alleles are not necessarily the only HLA alleles able to stimulate immunity to COA-1, and that the present invention extends to other sympathetic alleles.

Sympathetic HLA-II alleles are not necessarily present in all members of the human population but, where an individual has PBMC's (peripheral blood mononuclear cells) either autologous or allogeneic for either of these alleles, then it is sufficient simply to provide a vaccine comprising the immunising part of COA-1.

The immunising portion of COA-1 may be as much as the entire molecule, either with or without the mutation at position 399 but, more preferably, it simply comprises a peptide comprising at least the immunising epitope located between position 371 and 384 of the COA-1 transcript.

The immunising epitope may be presented in any suitable form. At its simplest, a vaccine comprising the peptide and a suitable carrier may be provided, together with, if required, any suitable excipients and/or adjuvants, for example.

The immunogenic peptide may also be presented in the form of nucleic acid in a form suitable for expression in the patient, either in a host organism, such as an attenuated virus, in a vaccine, or in the form of a suitable expression vector for expression *in vivo*.

It will be appreciated that the present invention extends to the sequence for COA-1, as well as the transcription product thereof. The invention further extends to the COA-1 sequence lacking one or more introns. The sequence of the invention may also lack one or more exons, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded. It is not necessary for the amino acid substitution at position 399 to be encoded, and it is generally preferred that this substitution is not encoded by the nucleotide sequences of the present invention. Without being bound by theory, it is possible that this substitution in the sequence of normal cells could affect the processing of the antigen, leading to a lack of expression of the immunogenic epitope. It will be appreciated that the degeneracy of the genetic code allows the nucleotide sequence to vary widely and still encode the

immunogenic sequence, but it is generally preferred to use the wild-type sequence, for simplicity, unless it is desired to engineer a splice site, for example.

Where the patient does not express a sympathetic HLA-II allele, then immunity may be conferred in a number of ways, any of which may also be employed in patients expressing a sympathetic allele.

Sympathetic alleles are expressed by PBMC's, such as B cells and fibroblasts. Thus, in one aspect, it is sufficient to isolate PBMC's or their progenitors from the patient and to transform these cells with HLA DRβ1*1301 or HLA DRβ1*0402 alleles, for example. Once successful transformation has been achieved, then the PBMC's, whether directly transformed, or whether obtained from the progenitors, may be used to stimulate the appropriate immunity, after reintroduction into the patient. This may be achieved either by introducing the PBMC's into the patient, followed by administration of a vaccine as described above, or the PBMC's may be contacted with COA-1, or a precursor therefor, or the immunising epitope or precursor therefor and, preferably once there has been some opportunity for endocytosis to occur, the treated PBMC's are administered to the patient. It will be appreciated that, in these circumstances, a "precursor" may include, for example, a fusion protein or a nucleic acid suitable for expression in the PBMC culture.

It will also be appreciated that suitable PBMC's may be obtained from, for example, a universal donor, and an immunising preparation may be made from such cells in a manner similar to that described above for transformed cells from the patients themselves.

It will be appreciated that the present invention extends to vaccines and immunising preparations as described above, as well as to host cells expressing COA-1, or a precursor therefor, provided that the immunising epitope is comprised in the transcript expressed thereby.

It will also be appreciated that the present invention extends to the use of antibodies recognising COA-1 mutated at position 399. Such antibodies may be used as a passive vaccine, for example or may be used in diagnostic assays for colorectal

cancer. Such assays may take the form of ELISA assays, for example, or may be used in suitable immunoblotting techniques.

Figure 1 shows a phenotypic characterization of the colorectal cancer line 1869 col.

Figure 1A shows a stained 1869 col cell line using antibodies directed against MHC class I (W6/32) and class II (L243) molecules, an epithelium marker (Ber-EP4), and the β subunit of prolyl-4-hydroxylase (5B5), a protein expressed exclusively in fibroblasts.

Figure 1B shows intracellular staining carried out using three cytokeratin reactive monoclonal antibodies: CK18, which reacts with cytokeratin 18; LP34, which reacts with multiple cytokeratins; and MNF116, which reacts with cytokeratins 5, 6, 8, 17 and probably 19.

Figure 1C shows staining of 1869 col cells at passage 6 (P6) and passage 20 (P20), carried out with the anti-CEA monoclonal antibody Col-1.

Figure 2 shows a cDNA clone isolated from the 1869 cDNA library encoding an antigen recognised by C111 T cells. The 293 cells expressing the MHC DRβ1*0402 or 1301 molecules were transfected with the 1D8 cDNA clone, or COA-1a, which corresponds to nucleotides 209-1318 of the COA-1 gene (see Figure 3). Target cells were either transfected with the COA-1a product alone or were co-transfected with a mixture of COA-1a and the full length HLA class II invariant chain (Ii). Additional targets were transfected with a control plasmid encoding GFP. Eighteen hours following the addition of 5×10^4 C111 T cells to the transfectants, supernatants were collected and IFN-γ release was measured by ELISA.

Figure 3 provides the sequence of the COA-1 gene isolated from the mRNA of the tumour line 1869 col. The COA-1 gene was isolated by RT-PCR from the 1869 col tumour cell line. The sequence of the 1D8 cDNA clone is shown in bold letters. The amino acid sequence corresponding to the T cell epitope is underlined, and the single nucleotide difference between the normal and tumour transcripts at position 1280 is noted. The amino acid sequence of the longest open reading frame in this transcript,

which is similar to the Socius gene product (20), is noted beneath the nucleotide sequence.

Figure 4 shows that the COA-1 transcript derived from normal B cells is not recognised by the clone C111 T cells. 293 cells expressing the indicated MHC DRβ1 molecules were transfected with COA-1a cDNAs isolated by RT-PCR from either the 1869 col cell line or from 1869 CD40L stimulated B cells. The GFP and Ii-1D8 constructs were used as negative and positive controls, respectively. Eighteen hours following the addition of 5×10^4 C111 T cells to the transfectants, supernatants were collected and IFN-γ release was measured by ELISA.

Figure 5 shows the relationship of the genetic sequence of COA-1 to the transcript, and the position of the primers used to generate the COA-1a product is shown by the arrows.

Several tumour reactive CD4$^+$ T lymphocytes were isolated from PBMC and TIL that were obtained following the establishment of autologous cultured colon tumour cell lines. These studies focused on a single clone of CD4$^+$ T cells, C111, that responded strongly to autologous tumour cells, and demonstrated low but significant reactivity with autologous EBV B cells, but failed to respond to autologous CD40L stimulated B cells. The gene encoding this antigen, termed COA-1, was isolated by screening an autologous cDNA library with clone C111 T cells. This gene appeared to be nearly identical to the gene encoding the human homologue of the rat Socius protein that was recently cloned using a yeast two-hybrid screening assay in which a member of the Rnd family of GTPases was used as bait (20). The Socius product was expressed at high levels in rat testis, but was expressed at significantly lower levels in rat lung, thymus and brain.

The longest open reading frame in the COA-1 transcript encodes a 437 amino acid product that corresponds to a portion of the human Socius gene product, and two overlapping peptides derived from this open reading frame were identified that could sensitise target cells expressing either HLA-DRβ1*0402 or 1301. The stimulation

observed with peptide pulsed targets was weak relative to that seen with the tumour cell lines that were recognised, and a minimum concentration of approximately 10 μM was needed to stimulate significant cytokine release from C111 T cells (Table 4).

Peptides derived from non-mutated tumour antigens such as tyrosinase (23) and TRP-1 or TRP-2 (17) have also been found to stimulate only relatively low levels of cytokine release from HLA class II-restricted, tumour reactive T cells, and minimal concentrations of between 1 and 10 μM of the peptides identified in these studies were required to sensitise target cells for T cell recognition. This may reflect the fact that these represent non-mutated self antigens, and that self tolerance results in the deletion of T cells that recognise peptides that bind to class II molecules with high affinity.

In addition, the autologous tumour cell line should present this peptide in the context of both the HLA-DRβ1*0402 and 1301 restriction elements, leading to enhanced stimulation of T cells reactive with this epitope. Transfectants expressing the COA-1 product stimulated significantly less cytokine release from C111 T cells than the autologous tumour cell line that had been induced to express high levels of HLA class II molecules. One potential explanation for this observation, however, is that the HLA class II positive 293 cells used as targets for transfection of the COA-1 gene products fail to express optimal levels of accessory molecules associated with the processing of this epitope.

The COA-1 transcript is nearly identical to sequences derived from a variety of tissues and tumour cell lines. These transcripts, however, comprise a large array of over 20 alternatively spliced products that are derived from at least 15 exons residing at the chromosome 1p36.1-p35 locus. The COA-1 product expressed in colon tumour cell lines appeared to contain a unique splicing pattern that did not correspond to any of the transcripts identified in the EST and GenBank databases, which may not encode products recognised by C111 T cells. Two nearly identical COA-1 gene products were amplified from EBV B cells, one of which was identical to that isolated from the colon tumour cells, and a second that contained a single nucleotide alteration at position 1280 that resulted in a substitution of a valine residue for the alanine residue at position 399

encoded by the dominant colon tumour cell product. It is not clear why C111 T cells only appeared to weakly recognise EBV B cells expressing the appropriate HLA class II gene products, but these observations could result from inherent differences in the antigen processing abilities of colon tumour cells and EBV transformed B cells.

Previous results have suggested that differences in the proteosomal subunits expressed by various cells may significantly influence antigen recognition, which provides one potential explanation for this finding (24). The RT-PCR products that were amplified from normal B cells and fibroblasts also appeared to uniquely encode the COA-1 variant that expressed a valine residue at amino acid 399, and target cells that were transfected with the COA-1 product that was amplified from normal cell lines were not recognised by C111 T cells.

Thus, it appears that normal B cells and fibroblasts either fail to express the COA-1 transcript that can be processed and presented to C111 T cells or express this product at only relatively low levels. The mechanisms involved in the preferential expression of these two transcripts are unknown, but these may represent the products of two nearly identical genes whose expression is differentially regulated. The correlation between expression of these products and the ability of C111 T cells to recognise the epitope encoded by these products provides further evidence that this represents the natural product recognised by these T cells and not a peptide mimic of the natural epitope.

An additional observation that is unexplained is how the alteration at position 399 affects recognition of the cell epitope comprised of amino acids 371 to 384 of the COA-1 transcript. Results of a previous study indicated that alteration of a distal residue can influence the ability of tumour reactive CD4+ T cells to recognise a mutated product of the CDC-27 gene product (21). Preliminary results presented in the prior study indicated that altered intracellular targeting of the mutated CDC-27 gene product may have played an important role in influencing processing of this gene product. Investigation of the cellular localization of the COA-1 protein in normal and tumour cells may help to indicate whether a similar mechanism may be involved with T cell recognition of this product.

Transfection studies, as well as peptide pulsing experiments indicated that either of the autologous HLA-DRβ1 alleles, DRβ1*0402 or DRβ1*1301 could present the T cell epitope to clone C111 T cells, which may potentially enhance the immunogenicity of this peptide in patient 1869 as well as other individuals that express these class II alleles. This observation is not unique, however, as examples of promiscuous recognition of class II and well as class I restricted epitopes have been noted in previous studies. In one report, CD4+ T cells were identified that also recognised an epitope of the herpes simplex type 2 virus virion protein, VP16 in the context of DRβ1*0402, 1102 or 1301 but not several closely related DR4, 11 or 13 subtypes (25). The sequences of the DRβ1*0402, 1102 and 1301 molecules are identical in a polymorphic region between amino acids 67 and 71, and site directed mutagenesis studies demonstrated that these residues were critical for the recognition of the viral epitope.

High levels of lymphocyte infiltration into tumours has been shown in some studies to be correlated with a good prognosis (26), but detailed investigations of the reactivity of infiltrating T cells have not been carried out. The expression of HLA class II molecules on colorectal cancer cells is also a favourable prognostic marker (27) (28). Previous studies resulted in the isolation of HLA class I (29) and class II (30, 31) restricted tumour reactive T cells from colon cancer patients, but only a limited panel of shared tumour specific antigens were identified in these studies. This report presents the description of an immunogenic CD4+ T cell epitope derived from a previously un-described colorectal cancer antigen that represents a potential target for immunotherapy in patients with this disease. Evaluation of the in vitro immunogenicity of the COA-1 epitope in DRβ1*0402 and *1301 positive colorectal patients may provide support for the use of this antigen in the immunotherapy of patients with this disease.

Example

Material and Methods

Cell lines and antibodies.

Colon cancer lines were generated from tumour liver metastases of five patients admitted to the Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. The cell lines were generated from the tumour samples by cutting the tissue into small fragments, followed by filtration through sterile gauze. The tumour cells were cultured in collagen-coated 6-well plates (Becton Dickinson, Franklin Lakes, NJ) in ACL-4 medium (InVitrogen, Carlsbard, CA) containing 10 % foetal bovine serum plus MEGM SingleQuots (Clonetics, Walkersville, MD) that contained epidermal growth factor (10 ng /ml), insulin (5 µg/ml), hydrocortisone (0.5 µg /ml), gentamicin (50 µg/ml), and amphotericin-B (50 ng/ml). Fresh medium was added to the cells every 5 days and fibroblasts were depleted from the cultures by carrying out a short-term treatment with trypsin. Immunofluorescent staining assays to assess cell surface HLA gene expression were carried out using the anti-class I mAb W6/32 and the anti-DR mAb L243 (Becton Dickinson).

The cell lines were stained using the mAb BerEP4 (DAKO, Cupertino, CA) that is directed against a cell surface molecule whose expression appears to be limited to epithelial tissues, and intracellular staining was carried out using the cytokeratin reactive mAbs CD18, LP34 and MNF116 (DAKO). Analysis of the expression of carcinoembryonic antigen (CEA), a molecule that is frequently over-expressed in colon tumour, was carried out using the mAb Col-1 (Zymed, South San Francisco, CA). The presence of fibroblasts in the cultured colon tumour cell lines was assessed using the mAb 5B5 (DAKO) that was directed against the β subunit of prolyl-4-hydroxylase, a protein involved with the synthesis of collagen. Flow cytometry was carried out using a FACScan (Becton Dickinson). The established colon cancer lines SW1463, SW480 and Colo205 were obtain from American Type Culture Collection (ATCC, Manassas, Virginia). The melanoma cell line 1681, the fibroblast cell line 1519 and the EBV-transformed B cell lines 1869 and 1519 were established in the Surgery Branch and

were cultured in RPMI plus 10 % FBS. The normal B cell lines 1847, 1681, 1872 and 1869 were generated, as previously described (16), by culturing PBL in ISCOVE's medium (InVitrogen) plus 10 % human serum in the presence of 100 IU/ml of CD40L (Immunex, Seattle, WA) and 100 IU/ml of recombinant human IL-4 (Pharmingen, San Diego, CA). The MHC class I and class II typing of the PBL and of the tumour lines used in this study was determined by single-stranded oligonucleotide probe–PCR typing carried out in the NIH HLA typing laboratory, and is summarised in Table 1. Antibodies used to carry out T cell receptor (TCR) analysis were obtained from Beckman/Coulter (Miami, FL) or Pierce/Endogen (Rockford, Il.).

Identification and characterization of tumour reactive T cells.

Tumour reactive T lymphocytes were generated from PBMC and tumour infiltrating lymphocytes (TIL) derived from colon cancer patients. Incubation of PBMC with autologous tumour cells that had been irradiated with 150 Gy was carried out at a tumour cell to lymphocyte ratio of 1 to 5 in RPMI media containing 300 IU/ml of recombinant human IL-2 plus 10 % human serum (HS). The cultures were stimulated weekly for a period of 5 to 6 weeks with autologous irradiated tumour cells. Cultures of TIL were established by initially plating fresh uncultured tumours at 5×10^5 cells per well in 24-well plates in RPMI containing 10 % HS and 1,000 IU/ml of IL-2. Tumour cells used for T cell stimulation were cultured for at least 10 days in RPMI containing 10 % HS to avoid the generation of T cells with reactivity against FBS. In addition, to optimise or up-regulate the expression of MHC molecules by tumour cells, these cells were incubated with IFN-γ (500 IU/ml) for 48 hr. The reactivity of the T cell lines against colon cancer lines was examined by incubation of 2×10^4 or, for some of the assays, 5×10^4 T cells in flat bottom 96-well plate in the presence of 5×10^4 autologous or allogeneic tumour cells. After overnight incubation at 37°C in 5 % CO_2, the supernatants were collected and T cell responses were evaluated using anti-IFN-γ antibodies (Endogen, Rockford, IL) in a sandwich ELISA assay.

After 3 weeks of culture the T cell lines were cloned by limiting dilution in the presence of allogeneic PBMC that had been irradiated with 50 Gy in RPMI media containing 30 ng/ml of OKT3 mAb in RPMI plus 10 % HS. The following day, fresh medium plus rh-IL-2 (300 IU/ml) was added to the cultures. After two weeks of culture, growth positive wells were screened for their ability to release IFN-γ in response to tumour stimulation. The T lymphocytes from sensitised PBMC that were chosen for further analysis, C4, C49 and C111, were isolated from cultures that were plated at 5 cells per well, but only 27% of the wells were positive for growth under these conditions, showing that some or all of these cells represent T cell clones.

Analysis carried out with antibodies directed against T cell receptor (TCR) families showed that greater than 95% of clone C4 T cells expressed a TCR reactive with an anti-Vβ5 reactive antibody, whereas C49 failed to express TCRs detected by any of the commercial antibodies. Amplification of the clone C111 TCR Vβ region product carried out using RT-PCR showed that this clone expressed a single sequence derived from the Vβ18 germline gene. Flow cytofluorimetric analysis showed that approximately 80% of C111 T cells expressed Vb18, but contaminating feeder cells used to expand the T cell clone may be responsible for the discrepancy between these results. Two CD4+ tumour reactive T cell cultures, C5 and C15, were also identified from 1869 TIL. These cultures were isolated from cells that were plated at one cell per well, and, as only 3% of the wells that were plated were positive for growth, these represent T cell clones. In addition, these cultures stained homogeneously with an antibody directed against Vb2, further showing that these represented T cell clones.

Tumour reactive cultures were then expanded in the presence of allogeneic PBL that were irradiated with 50 Gy in RPMI containing PHA (1μg/ml) and IL-2 (300 IU/ml). Immunofluorescent analysis of positive cultures was carried out using mAb directed against CD3, CD4, CD8, CD16, and CD56 (Becton Dickinson). Antibody blocking assays were carried out by pre-incubating target cells for 1 hour with W6/32, an antibody directed against a pan-MHC class I epitope, or L243, a mAb directed against a pan-HLA class II DR epitope. The T cells were then added to target cells, and IFN-γ release measured following an overnight incubation.

CIITA transduction of tumour lines.

In order to induce stable expression of cell surface MHC class II molecules, the tumour lines 1869 col, SW480, and Colo205 were transduced with a recombinant retrovirus that was generated by cloning the gene that encoded the human class II transactivator (CIITA) into the retroviral expression vector pCLRCX (17). The transduced 1869 tumour cells were then sorted using a FACSVantage™ cell sorter (Becton Dickinson) to obtain cells that homogeneously expressed relatively high levels of cell surface HLA class II expression.

Isolation of MHC class II DRβ1 molecules.

The DRβ1*0402 gene was isolated by carrying out an RT-PCR with RNA derived from the tumour line 1869 col, and the DRβ1*1301 gene was obtained by carrying out an RT-PCR with RNA derived from an autologous T cell line. Primers that were used to amplify HLA-DR were: 5'-TCCAGCATGGTGTGTCTGA-3' and 5'-CCTTGAATGTGGTCATCT-3'. Two additional primers were designed to specifically amplify the HLA-DR13 gene product: 5'CGTTTCTTGGAGTACTCTACGTC-3' and 5'-CCACCGCGGCCCGCTCGTCT-3'. The isolated products were cloned in the plasmid vector pCR-Blunt (Invitrogen, Carlsbard, CA) and sequenced using an ABI Prism 310 Genetic analyser (Perkin-Elmer, Shelton, CT). The genes were then cloned in the eukaryotic expression vectors pCDNA3.1 (Invitrogen) and the retroviral expression vector CLRCX4, discussed above.

Constructs encoding either of the HLA-DRβ1 genes were co-transfected along with a construct encoding the HLA-DRα gene into 293 cells. Stable transfectants were stained with the FITC labelled anti-HLA-DR mAb L243, and cells that were strongly positive for the expression of the cell surface HLA-DR molecules were isolated using a FACSVantage™ cell sorter (Becton Dickinson). To induce the expression of molecules involved with HLA class II antigen processing, such as the class II invariant chain,

MB genes, the 293 cells that had been transfected with the HLA-DR
:e then transduced with recombinant retroviral supernatants generated
C-CIITA construct, as previously described (17).

Library Construction and screening.

RNA was extracted from 1869 col tumour line using Triazol (GIBCO,
y (A) RNA was then isolated using poly (A) Tract (Promega, Madison,
y (A) RNA was then converted to cDNA using the SuperScript cDNA
(InVitrogen) and cloned in the episomal mammalian expression vector
BioSystems, Gaithersburg, MD). The pEAK8 vector had been modified
agment encoding amino acids one to 80 of the human invariant chain (Ii)
f the EF1-α promoter in order to express the cDNA inserts as fusion
target the gene products to the HLA class II antigen presentation
The recombinant cDNA was then electroporated into DH10B
ant cells (InVitrogen), and plasmid pools containing approximately 50
inants prepared as previously described (18). The 293 cell lines that were
th HLA-DRβ1*0402 (293-DR0402) or HLA-DRβ1*1301 (293-DR13)
ly transfected with DNA prepared from the cDNA pools (200 ng) using
2000 (InVitrogen) according to the manufacturer's directions.

r to conserve C111 T cells, screening assays were initially carried out by
mixture of $5x10^4$ 293-DR*0402 and $5x10^4$ 293-DR*1301 cells with
pools in 96 well flat bottom plates. The following day the cells were
10^5 cells T cells in AIM-V medium plus 2 % HS were added each well.
f incubation at 37^0C and 5 % CO_2, 100 µl of supernatant was collected
release was evaluated by ELISA. For subsequent assays, cDNA pools
:e transfected into 293 cells that expressed only a single HLA DR allele,
were tested for their ability to stimulate C111 T cells.

5' Rapid amplification of cDNA ends (RACE).

Total RNA was extracted from the 1869 col tumour cell line and a 5' RACE was performed using the Smart RACE cDNA amplification kit according the manufacturer's instructions (Clontech, Franklin Lakes, NJ). The RT-PCR products were cloned into the pCDNA 3.1 Topo cloning vector (Invitrogen) and recombinant DNA was prepared for sequence analysis. In addition, amplification of the full length COA-1 gene products was carried out using the Advantage 2 PCR kit (Clontech). The amplification was carried out by incubation at 95^0C for 1 minute, followed by 35 amplification cycles consisting of a 30 second incubation at 95^0C, a 30 second annealing step at 62^0C, and a 2 minute extension step at 68^0C.

Identification of T cell epitopes.

Peptides of 20 or 21 amino acids in length that overlapped by 15 amino acids that were encoded by the long open reading frame of the original cDNA clone that was isolated were synthesised by solid-phase method using a peptide synthesiser (AMS 422; Gilson Co., Inc. Middleton, WI). The purity of the peptides was verified by mass spectrometry (Tuft's Core Facility, Boston, MA). Allogeneic B cells (1×10^5 cells/well) that expressed either the $DR\beta1*0402$ or the $DR\beta1*1301$ molecules were incubated with 50 µg/ml in 100 µl/well of ISCOVE'S medium plus 10 % HS in flat bottom-96-well plates. After three hours, $1-5 \times 10^4$ T cells were added to the wells in 150 µl/well of medium and incubated for 18 hours at 37^0C and 5% CO_2, followed by measurement of INF-γ release by ELISA..

Results

Generation and characterization of colon cancer lines.

Cultured colon cancer lines were initially established from liver metastasis specimens obtained from five colorectal cancer patients. Analysis of one of the most rapidly proliferating cell lines that was obtained, 1869 col, demonstrated that these cells expressed a common epithelial marker, expressed cytokeratins associated with epithelial cells (Fig.1), and maintained a morphology in tissue culture that was typical of epithelial cells (data not shown).

In contrast, the cell lines did not stain with an antibody directed against the β subunit of prolyl-4-hydroxylase, a cell surface marker expressed in fibroblasts. Taken together, these results indicated that these cells were of epithelial origin and represented colon cancer cell lines and did not contain significant numbers of normal cells. The 1869 col cell line expressed uniform levels of MHC class I molecules and low or undetectable levels of cell surface MHC class II molecules were found on the same cells (Fig. 1), but treatment of the 1869 col cells with IFN-γ resulted in strong up-regulation of HLA class II expression (data not shown).

The carcinoembryonic antigen represents a marker that is expressed at high levels *in vivo* on colon tumour cells as well as on many colon tumour cell lines, but is not expressed by fibroblasts or hepatic cells. Analysis of 1869 col cells indicated that they expressed CEA (Fig. 1), and the additional colon tumour cell lines that were generated appeared to express similar levels of this gene product (data not shown). An early passage of the 1869 col cell line demonstrated high level expression of CEA, and lower but still significant levels of CEA expression were observed at later passages of 1869 col cells (Fig. 1). These observations are consistent with previous studies in which heterogeneous expression of CEA was observed on a variety of colon tumour cell lines (19).

Isolation and characterization of colon cancer reactive T lymphocytes.

In the initial attempts to derive colon tumour reactive T cells, tumour infiltrating lymphocytes (TIL) from patient 1869 were cultured in high dose IL-2. In addition, autologous tumour cells, that had been treated with IFN-γ to up-regulate HLA class II gene expression, were used to carry out in vitro mixed lymphocyte tumour cultures (MLTC) with PBMC from patient 1869. Three CD4$^+$ tumour reactive T cell clones, C4, C49 and C111, were initially selected for further analysis on the basis of their high degree of reactivity with the autologous tumour cell line.

The three clones derived from PBMC released IFN-γ in response to autologous tumour cells that had been treated with IFN-γ, and these clones released significantly higher levels of IFN-γ in response to 1869 tumour cells that had been treated with the CIITA and sorted for cells that constitutively expressed high levels of cell surface HLA class II molecules (Table 2).

Relatively low levels of IFN-γ were released following stimulation with the autologous 1869 EBV B cell line from the three T cell clones. All of the T cell clones released IFN-γ and GM-CSF but not IL-4 following stimulation with HLA class II positive tumour cells (data not shown), indicating that they represent cells of the Th1 cell phenotype.

In order to test whether the clones isolated from the PBMC recognised tumour cells in an MHC-restricted manner, cytokine release assays were carried out in the presence of anti-HLA class I and class II specific antibodies using stimulator cells bearing a variety of MHC haplotypes (Table 1). The results indicated that the C4, C49 and C111 T cell clones recognised the autologous tumour cells in the context of the HLA DR class II restriction element (Table 2). The C49 and C111 T cell clones also recognised the CIITA transduced allogeneic MHC class II$^+$ colon cancer lines SW480 and Colo 205 that shared expression of HLA-DRβ1*1301 with the autologous tumour, and this recognition was blocked by pre-incubation of the tumour cell lines with the anti-HLA-DR mAb.

Generally the responses were inhibited by between 50 and 90% by pre-incubation with the anti-HLA DR antibody, whereas less than 20% inhibition was observed with the anti-HLA class I antibody. The response of the C4 line to the SW480 CIITA treated tumour cell lines, as well as the response of C111 to theColo205 CIITA, were only partially inhibited by anti-HLA DR antibody, which might reflect the fact that these T cells can recognise additional ligands other than the classical TCR. The C4, C49 and C111 clones recognised autologous EBV B cells as well as an allogeneic EBV B cell line that shared expression of HLA DRβ1*1301 with autologous cells. Normal B cells that were generated by stimulating autologous PBMC with CD40 ligand plus IL-4, as well as an allogeneic fibroblast cell line that shared expression of HLA DRβ1*1301 with the 1869 col tumour and that was treated with IFN-γ to up-regulate HLA class II gene expression, stimulated little or no cytokine release from these T cells (Table 3).

Two CD4+ T cell clones from TIL 1869 that responded in preliminary assays to autologous HLA class II positive tumour cells were also tested for their ability to recognise autologous as well as allogeneic colon tumour cell lines. Clones C4, C49 and C111, as well as two clones derived from 1869 TIL, C5 and C15, responded to the allogeneic colon tumour cell line 1847 col that shared expression of the HLA-DRβ1*1301 gene product with the autologous tumour. In contrast, the allogeneic 1872 col cell line that did not share expression of any HLA DR gene products with the 1869 col tumour failed to stimulate significant cytokine release from the T cell clones.

Identification of the antigen recognised by C111 T cells.

Further studies aimed at identifying tumour antigens expressed on 1869 col cells focused on C111 T cells, which was the only T cell clone that expanded sufficiently to allow the cDNA library to be screened. The results of studies carried out with additional tumour histologies indicated that C111 T cells did not recognise two allogeneic renal cell lines, as well as a prostate tumour cell line that shared expression of HLA-DRβ1*1301 with the 1869 col cell line (data not shown). A single allogeneic

melanoma cell line that expressed HLA-DRβ1*0402 was identified, 1681 mel. Cell surface HLA class II expression was up-regulated following treatment of the 1681 mel cell line with IFN-γ, and the treated cells were recognised by C111 T cells, indicating that certain tumour types shared expression of the antigen recognised by these T cells (Table 3).

Stable transfectants of the 293 cell line that expressed either the autologous MHC class II DRβ1*0402 or 1301 gene products molecules were then mixed in equal numbers and transiently transfected with DNA pools generated from the autologous tumour cell cDNA library. The positive pool that was initially identified following the screening of approximately 3×10^4 clones, 4G3, appeared to sensitise either 293-DRβ1*0402 or 1301 target cells for recognition by C111 T cells, and a single cDNA clone that could sensitise target cells for recognition by C111 T cells, 1D8, was identified (Fig. 2).

An assay carried out by transfection of the 293-DRβ1*0402$^+$ or 1301$^+$ cell lines individually with the 1D8 cDNA indicated that either of these HLA class II restriction elements could present the T cell epitope to C111 T cells. In contrast, 293 cell lines that expressed the HLA-DRβ1*0101, 0401, 0701 or 1601 class II alleles failed to stimulate these T cells following transfection of the 1D8 cDNA clone (data not shown), indicating that presentation of this epitope to C111 T cells may be limited to the two autologous HLA-DR alleles expressed by 1869 col cells. Further screening of the cDNA library resulted in the isolation of a second cDNA clone that was nearly identical to the 1D8 clone. The isolation of a second clone with a nearly identical sequence supports the finding that this represents the natural transcript encoding the antigen recognised by C111 T cells

Characterization of colorectal tumour associated antigen COA-1.

The 1D8 insert contained a 44 bp polyA tail at the 3' end, but appeared to represent a partial cDNA clone as it was only 291 bp in length. The 5' end of the gene

product that was expressed in the 1869 col cell line was then isolated by carrying out a rapid amplification of cDNA ends (RACE) reaction using nested internal primers complementary to the sequence of the 1D8 clone. Sequencing of products that were cloned from this reaction indicated that a 1412 bp product represented the predominant transcript of the gene in the 1869 col cell line that encoded the antigen recognised by C111 T cells, which was designated colorectal antigen-1 (COA-1) (Fig 3).

Comparison of the COA-1 sequence with the genomic DNA sequence database indicated that this product was derived from 13 exons, but at least two additional alternatively spliced products of this gene were isolated from the RACE reaction. An alignment of the COA-1 transcript with the human EST database indicated that this was identical or nearly identical to several sequences obtained from normal human brain, placenta, ovary, and testis, as well as sequences obtained from a variety of adenocarcinomas.

The 5' end of the transcript cloned from the RACE reaction corresponded to the 5' end of several EST sequences found in the database, and the 3' end of the original cDNA clone corresponded to the 3' end of the EST transcripts derived from several cell lines, indicating that these may represent the authentic 5' and 3' ends of the predominant COA-1 colon tumour cell transcript. The COA-1 sequence was also nearly identical to that of a transcript encoding the human homologue of the rat Socius protein, a molecule that was recently cloned on the basis of its ability to bind to a member of the Rnd family of GTPases (20).

Forward and reverse primers located at or near the 5' and 3' ends of the putative COA-1 gene product were then used to carry out an RT-PCR from 1869 RNA, as the RACE products that had been cloned only comprised a portion of the normal transcript. When RT-PCR was carried out with several primers that were proximal to the putative 5' end of the transcript in combination with primers that were complementary to the highly repetitive G/C rich sequence near to the 3' end of the COA-1 transcript, a variety of non-specific transcripts were generated (data not shown). A product that was designated COA-1a was, however, successfully amplified from 1869 col RNA using

two primers that encompassed the region between nucleotides 290 and 1318 of the putative full length COA-1 transcript.

Transfectants that co-expressed the COA-1a gene along with either HLA-DRβ1*0402 or 1301, appeared to stimulate comparable levels of cytokine release from C111 T cells to those transfected with the truncated 1D8 cDNA clone, showing that the full length gene can be processed relatively efficiently (Fig. 2). Co-transfection of the COA-1a gene with a construct encoding the full length human invariant chain (Ii) had little or no effect on the recognition of target cells transfected with the COA-1a product by C111 T cells. Thus, either the levels of Ii expression in 293 cells that were also transfected with a construct encoding the CIITA gene product was adequate for recognition of this epitope, or Ii expression does not have a significant impact on the processing of the COA-1 epitope.

In addition, the COA-1a product was not fused with amino acids one to 80 of the · human Ii molecule, which had previously been shown to enhance the recognition of some HLA class II antigens (21).

The observation that the fusion of the cDNA clone with the invariant chain did not enhance recognition by the CD4+ T cells shows that the COA-1 antigen may naturally target the endogenous HLA class II processing pathway in colon tumour cells.

The expression pattern of the COA-1 gene was then examined in several colorectal, melanoma, and EBV-B cell lines, as well as in several normal cell lines which included CD40L stimulated B cell and fibroblast cell lines. The results of Northern blot analysis indicated that this gene was expressed at relatively low levels in colon and melanoma tumour cell lines, EBV B cells, normal B cells and fibroblasts, and quantitative TaqMan RT-PCR indicated that the levels of expression did not differ significantly between these cells (data not shown).

The observation that the level of expression of the COA-1 gene did not differ significantly between cell lines that were or were not recognised by C111 T cells, showed that these cells express similar but non-identical products. Therefore,

transcripts of the COA-1 gene that were expressed in the autologous and allogeneic CD40L stimulated B cells, as well as allogeneic fibroblast cell lines, were isolated using RT-PCR and sequenced.

The results of sequencing carried out with the bulk RT-PCR products showed that CD40L stimulated B cells and fibroblast cell lines predominantly expressed products that appeared to be identical to the COA-1 transcript derived from 1869 col cells with the exception of a single substitution of a T for a C residue at nucleotide position 1280, resulting in a change at amino acid 399.

The COA-1 transcripts that were expressed in CD40L B cells were isolated by carrying out RT-PCR and cloning the resultant products. Ten out of ten clones from the CD40L B cells that were sequenced contained a T at position 1280 but were otherwise identical to the 1869 col COA-1 transcript.

Amplification of the COA-1 gene product from allogeneic colorectal tumour lines SW1463, SW480 and 1847 col, as well as the 1681 mel line, showed that these cells predominantly expressed products containing a C residue at position 1280, as determined by sequencing the bulk, un-cloned RT-PCR products that were amplified from these cells (data not shown). Two peaks of comparable heights that corresponded to C and T residues at position 1280 of the COA-1 transcript were derived by sequencing the un-cloned RT-PCR product from autologous EBV B cells, indicating that these products may be expressed at similar levels in these cells. The results obtained using RNA from autologous CD40L stimulated B cells, EBV B cells, and the colon tumour cell lines were confirmed by repeated analysis carried out on products obtained from four independent RT-PCR reactions, showing that the residue found at nucleotide 1280 of the COA-1 transcripts did not represent a PCR mutation (data not shown).

To evaluate the significance of the single base pair change at position 1280 in the COA-1a sequence, the RT-PCR products obtained from autologous CD40L stimulated B cells were cloned in a eukaryotic expression vector. A plasmid containing the COA-1a transcript that was amplified from the normal B cells was then compared

with products cloned from 1869 col cells for its ability to sensitise 293-DR*0402 or 293-DR*1301 cells for recognition by C111 T cells. Target cells expressing either of the autologous HLA-DR genes that were transfected with the COA-1a or 1D8 gene products, but not the product that was isolated from CD40L activated B cells, stimulated cytokine release from C111 T cells (Fig. 4). These results showed that there was a correlation between the recognition of normal B cells and tumour cells and the ability of the COA-1 gene products that were expressed by these cells to sensitise targets for recognition by C111 T cells.

Identification of the epitope recognised by the CD4^{+} clone C111.

The results of transfection studies carried out using truncated COA-1 gene products showed that the C111 T cell epitope was encoded by a region located between nucleotides 1121 and 1288 of the COA-1 transcript. The longest open reading frame in the COA-1 transcript, which overlapped with the Socius gene product (20), was utilised as the basis for the synthesis of peptides that were used to identify the T cell epitope recognised by C111 T cells.

Peptides that were 20 or 21 amino acids in length and that overlapped by either 14 or 16 amino acids, were than synthesised and tested for their ability to sensitise target cells for recognition by C111 T cells. Since autologous normal B cells could not be efficiently expanded, allogeneic normal B cells expressing either DRβ1*0402 or DRβ1*1301 were used to carry out these assays.

The 1681 and 1847 CD40L stimulated normal B cell lines shared expression of HLA-DRβ1*0402 and HLA-DRβ1*1301 molecules, respectively, with the autologous tumour cell line. These cells were incubated with the panel of peptides and then tested for their ability to stimulate cytokine release from C111 T cells. The results showed that 1681 and 1847 CD40L B cells that were pulsed with either of the two overlapping peptides FSTFPPTLYQDDTLTLQAAG and TLYQDDTLTLQAAGLVPKAA stimulated significant cytokine release from C111 T cells.

These T cells thus recognise the peptide TLYQDDTLTLQAAG, which represents the overlapping region in these peptides. The L at position two, the T at the position 7 and L at position 10 in this sequence conform to an HLA binding motif that has been identified for the HLA-DRβ1*0402 class II allele (22). However, it was not possible to identify the potential anchor residues in this sequence that were involved in binding to the HLA- DRβ1*1301 allele. Nevertheless, these observations show that C111 T cells recognise a single peptide epitope in the context of either the HLA-DRβ1*0402 or 1301 class II gene products.

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Table 1 MHC Haplotype of cell lines

	A	B	C	DRβ1	DRβ3-5	DQ
1869	3,24	35,38	0401,1203	0402,1301	3*01,4*01	03,06
1870	24	35	04	1202	3*03	03
1872	02, 03	07, 4402	0501, 0702	0401,1501	4*01, 5*01	03, 06
1681	01,0201	08,44	N.D.	0301,0402	3*0101,4*01	0301,0402
1847	02	18, 44	05, 0701	0401,1301	3*01, 4*01	03, 06
1519	24,32	1401,4402	05,08	0701,1301	3*01,4*01	02, 06

Table 2 Specific recognition of colon cancer lines by CD4^{+} clones from patient 1869.

Target cells	Antibody[a]	HLA-DRβ1	T cell[b]		
			C4	C49	C111
None	None		<8[c]	<8	<8
1869 col + IFN-γ[d]	None	*0402, *1301	234	1213	536
	W6/32		212	1100	442
	L243		107	97	17
1869 col CIITA	None	*0402, *1301	536	5178	5005
	W6/32		527	4987	4249
	L243		47	254	305
1870 col + IFN-γ	None	*1202	<8	<8	<8
	W6/32		<8	<8	<8
	L243		<8	<7.8	<8
1872 col + IFN-γ	None	*0401, *1501	<8	<8	<8
	W6/32		<8	<8	<8
	L243		<8	<8	<8
SW 480 CIITA	None	*0103, *1301	879	968	963
	W6/32		780	902	996
	L243		571	129	127
Colo 205 CIITA	None	*0401, *1301	68	942	686
	W6/32		76	951	669
	L243		78	170	489
1869 EBV-B		*0402, *1301	52	126	322

a. Target cells were pre-incubated for 1 hour with either the anti-MHC class I mAb W6/32 or the anti-HLA DR mAb L243 before addition to T cells.
b. 2x10⁴ T cells were incubated with 5x10⁴ target cells in flat bottom 96-well plate in 250 μl of AIMV 2% HS. After 18 hrs. the supernatants IFN-γ secretion was evaluated by ELISA.
c. pg/ml of IFN-γ.
d. Where indicate, target cells were pre-incubated for 48 hrs with 500 IU of IFN-γ.

Table 3 CD4^{+} clones recognised colon cancer lines but not normal B or fibroblast cells sharing MHC class II molecules

A

Stimulator	Antibody[a]	HLA-DRβ1	T cell[b]				
			TIL		PBL		
			C5	C15	C4	C49	C111
None	None		<8[c]	<8	<8	<8	<8
1869 col CIITA	None	*0402,	8695	1259	12328	12749	15269
	L243[c]		279	162	511	524	790
1847 col + IFN-γ[d]	None	*0401,	2008	457	598	9758	11576
	L243		2055	327	585	790	2938
1872 col + IFN-γ	None	*0401,	72	<8	61	<8	66
	L243		75	<8	60	<8	41
1869 EBV-B	None	*0402,	79	116	122	232	209
1519 EBV-B	None	*0701,	112	24	99	106	220
1519 Fibroblast +	None	*0701,	<8	<8	<8	55	62
1869 CD40LB[e]	None	*0402,	<8	<8	<8	<8	45

B

Stimulator	Antibody[a]	T cell[b]
		C111
None	—	23
1869 col CIITA	-	15269
1869 col CIITA	HLA-DR[c]	790
1681 mel+ IFN-γ	-	10298
1681 mel+ IFN-γ	HLA-DR	253
1869 B cells	-	65
1681 B cells	-	22

a. Where indicated, target cells were pre-incubated for 1 hour with the anti-HLA DR mAb L243.
b. 2×10^4 of the indicated T cells were incubated with 5×10^4 target cells in flat bottom 96-well plate in 250 µl of AIMV 2% HS. After 18 hrs. the supernatants IFN-γ secretion was evaluated by ELISA.
c. pg/ml of IFN-γ.
d. Where indicated, target cells were pre-incubated for 48 hrs with 500 IU of IFN-γ.
e. B cells from the patient 1869 were in vitro cultured with CD40L (100 IU/ml) and IL-4 (100 IU/ml).

Table 4 Identification of the COA-1-derived epitopes recognised by the CD4^{+} clone C111.

Stimulator	HLA-DRβ1	No peptide
None		<8[a]
1869 col	0402, 1301	2186
1681 CD40LB	0301, 0402	<8
1847 CD40LB	0401, 1301	<8

Stimulator	HLA-DRβ1	Peptide[b]	Peptide Conc. (μg/ml)				
			100	50	25	12.5	6.25
1681 CD40LB	0301, 0402	FSTFPPTLYQDDTLTLQAAG	105	236	69	<7.8	<7.8
1681 CD40LB		TLYQDDTLTLQAAGLVPKAA	51	159	<7.8	<7.8	<7.8
1681 CD40LB		DDTLTLQAAGLVPKAALLLRA	11	16	<7.8	<7.8	<7.8
1681 CD40LB		LQAAGLVPKAALLLRARRAP	21	12	<7.8	<7.8	<7.8
1847 CD40LB	0401, 1301	ASAFEIFSTFPPTLYQDDTL	<7.8	<7.8	<7.8	<7.8	<7.8
1847 CD40LB		FSTFPPTLYQDDTLTLQAAG	226	397	296	79	<7.8
1847 CD40LB		TLYQDDTLTLQAAGLVPKAA	79	326	<7.8	<7.8	<7.8
1847 CD40LB		DDTLTLQAAGLVPKAALLLRA	22	33	<7.8	<7.8	<7.8
1847 CD40LB		LQAAGLVPKAALLLRARRAP	52	32	<7.8	<7.8	<7.8

a. The CD4^{+} T cell clone C111 was the added at 2x10^{4} cells/well at the final volume of 250 μl/well of ISCOVE's plus 10 % HS and after 18 hrs. of incubation the supernatants were collected and the IFN-γ release was evaluated by ELISA.

b. Peptides of 20 or 21 amino acids overlapping by 15 amino acids were synthesised using the putative COA-1 protein, in the 1D8 region (1012-1318 bp). 4x10^{5}/ml of B cells sharing one of the DRβ1 molecules (*0402 or *1301) with the autologous tumour 1869, were incubated for three hrs. at 37°C and 5% CO_2 in the presence or not (-) of the peptides at the final volume of 100 μl/well in ISCOVE's plus 10% HS.

CLAIMS:

1.   A method for stimulating immunity against colorectal cancer, comprising stimulating the production of antibodies against the human equivalent of the Socius gene product, wherein the alanine residue at position 399 is substituted by a valine residue, wherein either or both of the coding sequence and the transcript thereof is as shown in Figure 5.

2.   A method for stimulating immunity against a substance as defined in claim 1, comprising the administration of an epitope located between amino acids 371 and 384, inclusive, of the COA-1 transcript in an immunity-stimulating form to the patient.

3.   A method according to claim 1 or 2, wherein immunity is stimulated by a peptide comprising the sequence TLYQDDTLTLQAAG.

4.   A method according to claim 3, wherein the peptide is an oligopeptide.

5.   A method according to any preceding claim, wherein immunity is stimulated in the patient in conjunction with PBMC's allogeneic or autologous for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

6.   A method according to claim 5, wherein the allele is selected from HLA DRβ1*0402 and HLA DRβ1*1301.

7.   A method according to any preceding claim, wherein the patient has PBMC's autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner, the method comprising administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any preceding claim, to the patient.

8.   Use of an immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, in the preparation of a medicament for the treatment or prophylaxis of colorectal cancer, wherein the patient has PBMC's autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

9.   A method for stimulating immunity to colorectal cancer in a patient, said method comprising

i) isolating PBMC's or their progenitors from the patient and transforming said cells with at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner,

ii) introducing the transformed PBMC's back into the patient, and

iii) administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, to the patient.

10.   A method according to claim 9, wherein the immunising portion of COA-1, or a precursor therefor, is administered with the transformed PBMC's.

11.   A method for stimulating immunity to colorectal cancer in a patient, said method comprising administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, to the patient, in conjunction with PBMC's obtained from a universal, or other suitable, donor for the patient, wherein said PBMC's are autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

12.   A vaccine as defined in any preceding claim.

13.   A nucleic acid encoding all or part of COA-1, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded.

14.   A nucleic acid encoding COA-1 wherein the alanine residue at position 399 is substituted by a valine residue, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded.

15.   The transcription product of a nucleic acid according to either of claims 13 and 14.

16.   A host cell expressing COA-1, or a precursor therefor, provided that the immunising epitope is comprised in the transcript expressed thereby.

17.   An antibody recognising COA-1 mutated at position 399.

18.   A passive vaccine comprising an antibody according to claim 17.

19.   A diagnostic assay for colorectal cancer comprising an antibody according to claim 17.

ABSTRACT

COLORECTAL ANTIGEN

A point mutation in a novel, commonly expressed gene, designated as COA-1 herein, is diagnostic of colorectal cancer.

Fig.1A

Fig 1B

Fig 1C

Fig 2

Figure 3

Nucleotide sequence of COA-1 (the gCc triplet comprising C at nucleotide position 1280, encodes Alanine)

```
cgctgcggga cggctagcgg ccctgcgtgg aggcgaggaa tccgcatcta tggagatgtc    60
cctgcatccc atgactcgga gctgatggcc ttcatgacga ggaagttgtg ggacctggag   120
cagcaggtga aggcccagac tgatgagata ctgtccaagg atcagaagat agcggcccta   180
gaggacctgg tgcagaccct ccggccacac ccagccgagg caaccctgca gcggcaggag   240
gaactggaga cgatgtgtgt gcagctgcag cggcaggtca gggagatgga gcggttcctc   300
agtgactatg gcctgcagtg ggtgggcgag cccatggacc aggaggactc agagagcaag   360
acagtctcag agcatggcga gagggactgg atgacagcca agaagttctg gaagccaggg   420
gactcattgg cgcccctga ggtggacttt gacaggctgc tggccagcct gcaggatctt    480
agtgagctgg tggtagaggg tgacacccaa gtgacaccag tgcccggcgg ggcacggctg   540
cgtaccctcg agcccatccc gctgaagctc taccggaatg gcatcatgat gttcgacggg    600
cccttccagc ccttctacga tccctccaca cagcgctgcc tccgagacat attggatggc    660
ttctttccct cagagctcca gcgactgtac cccaatgggg tccccttaa ggtgagtgac     720
ttgcgcaatc aggtctacct ggaggatgga ctggacccct tcccaggcga gggccgtgtg    780
gtgggcaggc agcggatgca caaggccttg gacagggtgg aggagcaccc aggctccagg    840
atgactgctg agaaatttct gaacaggctc cccaagtttt gatccggcaa ggcgaggtga    900
ttgacatccg gggccccatc agggacacct tgcagaactg ctgcccattg cctgccgga    960
tccaggagat tgtggtggag acgcccacct tggccgctga gcgagagagg agccaggagt   1020
cacccaacac accggcaccc ccgctctcca tgctgcgcat caagtctgag aatggggaac   1080
aggccttcct actgatgatg cagcctgaca acaccattgg ggacgtgcga gctctgctag   1140
cgcaggccag ggtcatggat gcctctgcct ttgagatctt cagcacattc ccgcccaccc   1200
tctaccagga cgatacactc acgctgcagg ctgcaggcct tgtgcccaaa gcagcactgc   1260
tgctgcgggc acgccgagcc ccgaagtcca gcctgaaatt cagtcctggt ccctgtcccg   1320
gtcccggtcc cggccccagt cccggtcccg gtcccggctc cagtccctgt cccggaccca   1380
gtcccagccc ccaataaagc acccacccc tc                                  1412
```

Protein Sequence of COA-1 (comprising Alanine at position 399)

```
MAFMTRKLWD  LEQQVKAQTD  EILSKDQKIA  ALEDLVQTLR  PHPAEATLQR  QEELETMCVQ    60
LQRQVREMER  FLSDYGLQWV  GEPMDQEDSE  SKTVSEHGER  DWMTAKKFWK  PGDSLAPPEV   120
DFDRLLASLQ  DLSELVVEGD  TQVTPVPGGA  RLRTLEPIPL  KLYRNGIMMF  DGPFQPFYDP   180
STQRCLRDIL  DGFFPSELQR  LYPNGVPFKV  SDLRNQVYLE  DGLDPFPGEG  RVVGRQRMHK   240
ALDRVEEHPG  SRMTAEKFLN  RLPKFVIRQG  EVIDIRGPIR  DTLQNCCPLP  ARIQEIVVET   300
PTLAAERERS  QESPNTPAPP  LSMLRIKSEN  GEQAFLLMMQ  PDNTIGDVRA  LLAQARVMDA   360
SAFEIFSTFP  PTLYQDDTLT  LQAAGLVPKA  ALLLRARRAP  KSSLKFSPGP  CPGPGPGPSP   420
GPGPGSSPCP  GPSPSPQ                                                      437
```

Alanine at position 399

```
          10        20        30        40        50        60
CGCTGCGGGACGGCTAGCGGCCCTGCGTGGAGGCGAGGAATCCGCATCTATGGAGATGTC

          70        80        90        100       110       120
CCTGCATCCCATGACTCGGAGCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAG
CAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAAGGGCTGATGGCCTTCATGACGA
GGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAAGG
GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGAC
TGATGAGATACTGTCCAAGGATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGACCCT
CCGGCCACACCCAGCCGAGGCAACCCTGCAGCGGCAGGAGGAACTGGAGACGATGTGTGT
GCAGCTGCAGCGGCAGGTCAGGGAGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAGTG
GGTGGGCGAGCCCATGGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGA
GAGGGACTGGATGACAGCCAAGAAGTTCTGGAAGCCAGGGGACTCATTGGCGCCCCCTGA
GGTGGACTTTGACAGGCTGCTGGCCAGCCTGCAGGATCTTAGTGAGCTGGTGGTAGAGGG
TGACACCCAAGTGACACCAGTGCCCGGCGGGGCACGGCTGCGTACCCTCGAGCCCATCCC
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGA
TCCCTCCACACAGCGCTGCCTCCGAGACATATTGGATGGCTTCTTTCCCTCAGAGCTCCA
GCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGACTTGCGCAATCAGGTCTACCT
GGAGGATGGACTGGACCCCTTCCCAGGCGAGGCCGTGTGGTGGGCAGGCAGCGGATGCA
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTTCT
GAACAGGCTCCCCAAGTTTTGATCCGGCAAGGCGAGGTGATTGACATCCGGGGCCCCATCAGGGACACCTT
GCAGAACTGCTGCCCATTGCCTGCCCGG
ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGCTGAGCGAGAGAGGAGCCAGGAGTCACCCAACAC
ACCGGCACC
CCCGCTCTCCATGCTGCGCATCAAGTCTGAGAATGGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACA
ACACCATTG
GGGACGTGCGAGCTCTGCTAGCGCAGGCCAGGGTCATGGATGCCTCTGCCTTTGAGATCTTCAGCACATTC
CCGCCCACC
CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGGCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGC
ACGCCGAGC
CCCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCCTGTCCCGGTCCCGGTCCCGGCCCCAGTCCCGGTCCCG
GTCCCGGCT
CCAGTCCCTGTCCCGGACCCAGTCCCAGCCCCCAATAAAGCACCCACCCCCTC
SSPCPGPSPSPQ
```

```
          10        20        30        40        50        60
cgctgcgggacggctagcggccctgcgtggaggcgaggaatccgcatctatggagatgtc
          70        80        90        100       110       120
cctgcatcccatgactcggagctgatggccttcatgacgaggaagttgtgggacctggag
          130       140       150       160       170       180
cagcaggtgaaggcccagactgatgagatactgtccaaggatcagaagatagcggcccta
          190       200       210       220       230       240
gaggacctggtgcagaccctccggccacacccagccgaggcaaccctgcagcggcaggag
          250       260       270       280       290       300
gaactggagacgatgtgtgtgcagctgcagcggcaggtcagggagatggagcggttcctc
          310       320       330       340       350       360
agtgactatggcctgcagtgggtgggcgagcccatggaccaggaggactcagagagcaag
          370       380       390       400       410       420
acagtctcagagcatggcgagagggactggatgacagccaagaagttctggaagccaggg
          430       440       450       460       470       480
gactcattggcgccccctgaggtggactttgacaggctgctggccagcctgcaggatctt
          410       420       430       440       450       460
agtgagctggtggtagagggtgacacccaagtgacaccagtgcccggcggggcacggctg
          470       480       490       500       510       520
```

```
cgtaccctcgagcccatcccgctgaagctctaccggaatggcatcatgatgttcgacggg
        530       540       550       560       570       580
cccttccagcccttctacgatccctccacacagcgctgcctccgagacatattggatggc
        590       600       610       620       630       640
ttctttccctcagagctccagcgactgtaccccaatggggtccccttttaaggtgagtgac
        650       660       670       680       690       700
ttgcgcaatcaggtctacctggaggatggactggaccccttcccaggcgagggccgtgtg
        710       720       730       740       750       760
gtgggcaggcagcggatgcacaaggccttggacagggtggaggagcacccaggctccagg
        770       780       790       800       810       820
atgactgctgagaaatttctgaacaggctcccccaagtttttgatccggcaaggcgaggtga
        830       840       850       860       870       880
ttgacatccggggccccatcagggacaccttgcagaactgctgcccattgcctgcccgga
        890       900       910       920       930       940
tccaggagattgtggtggagacgcccaccttggccgctgagcgagagaggagccaggagt
        950       960       970       980       990      1000
cacccaacacaccggcaccccgctctccatgctgcgcatcaagtctgagaatggggaac
       1010      1020      1030      1040      1050      1060
aggccttcctactgatgatgcagcctgacaacaccattggggacgtgcgagctctgctag
       1070      1080      1090      1100      1110      1120
cgcaggccagggtcatggatgcctctgcctttgagatcttcagcacattcccgcccaccc
       1130      1140      1150      1160      1170      1180
tctaccaggacgatacactcacgctgcaggctgcaggccttgtgcccaaagcagcactgc
       1190      1200      1210      1220      1230      1240
tgctgcgggcacgccgagccccgaagtccagcctgaaattcagtcctggtccctgtcccg
       1250      1260      1270      1280      1290      1300
gtcccggtcccggccccagtcccggtcccggtcccggctccagtccctgtcccggaccca
       1370      1380      1390      1400      1410
gtcccagcccccaataaagcacccaccccctc
       1370      1380      1390      1400      1410
```

MAFMTRKLWDLEQQVKAQTDEILSK

DQKIAALEDLVQTLRPHPAEATLQRQE

ELETMCVQLQRQVREMERFLSDYGLQW

VGEPMDQEDSESKTVSEHGERDWMTA

KKFWKPGDSLAPPEVDFDRLLASLQDL

SELVVEGDTQVTPVPGGARLRTLEPIP

LKLYRNGIMMFDGPFQPFYDPSTQRC

LRDILDGFFPSELQRLYPNGVPFKVSD

LRNQVYLEDGLDPFPGEGRVVGRQRMH

KALDRVEEHPGSRMTAEKFLNRLPKF

VIRQGEVIDIRGPIRDTLQNCCPLPAR

IQEIVVETPTLAAERERSQESPNTPAP

PLSMLRIKSENGEQAFLLMMQPDNTI

GDVRALLAQARVMDASAFEI**FSTFPPT**

LYQDDTLTLQAAGLVPKAALLLRARRA
                    t
V

COA-1a

←

PKSSLKFSPGPCPGPGPGPSPGPGPG

Figure 4

```
                    10        20        30        40        50        60        70        80
CGCTGCGGACGGCTAGCGCCCTGCGTGGAGGCGAGGAATCCGCATCTATGGAGATGTCCCTGCCATCCCATGACTCGGA

                    90       100       110       120       130       140       150       160
GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGTGAAGGCCCAGACTGATGAGATACTGTCCAAGG
 M  A  F  M  T  R  K  L  W  D  L  E  Q  Q  V  K  A  Q  T  D  E  I  L  S  K

                   170       180       190       200       210       220       230       240
ATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGAGACCCTCCGGCCACACCAGCCAGCACCTGCAGCGGCCAGGAG
 D  Q  K  I  A  A  L  E  D  L  V  Q  T  L  R  P  H  P  A  E  A  T  L  Q  R  Q  E

                   250       260       270       280       290       300       310       320
GAACTGGAGACGATGTGTGTGCAGCTGCAGCGGCAGGTCAGGGAGGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAGTG
 E  L  E  T  M  C  V  Q  L  Q  R  Q  V  R  E  M  E  R  F  L  S  D  Y  G  L  Q  W
                                                      COA-1a

                   330       340       350       360       370       380       390       400
GGTGGGCGAGCCCATGGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGGACTGGATGACAGCCA
 V  G  B  P  M  D  Q  E  D  S  E  S  K  T  V  S  E  H  G  E  R  D  W  M  T  A

                   410       420       430       440       450       460       470       480
AGAAGTTCTGGAAGCCAGGGGACTCATTGGCGCCCCCTGAGGTGGACTTTGACAGGTGCTGGCCAGCCTGCAGGATCTT
 K  K  F  W  K  P  G  D  S  L  A  P  P  E  V  D  F  D  R  L  L  A  S  L  Q  D  L

                   490       500       510       520       530       540       550       560
AGTGAGCTGGTGGTAGAGGGTGACACCCAGGTGACCAGTGCCCGGCGGGGGGCACGCGTGCCTGCTACCCTGCAGCCCATCCC
 S  E  L  V  V  E  G  D  T  Q  V  T  P  V  P  G  G  A  R  L  R  T  L  E  P  I  P

                   570       580       590       600       610       620       630       640
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCCTCCACACAGCGCTGCC
 L  K  L  Y  R  N  G  I  M  M  F  D  G  P  F  Q  P  F  Y  D  P  S  T  Q  R  C

                   650       660       670       680       690       700       710       720
TCCGAGACATATTGGATGGCTTCTTTCCTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGAC
 L  R  D  I  L  D  G  F  F  P  S  E  L  Q  R  L  Y  P  N  G  V  P  F  K  V  S  D

                   730       740       750       760       770       780       790       800
TTGCGCAATCAGTCTACCTGGGAGGATGGACTGGACCCCTTCCCAGGCCGAGGGCGAGGGCGTGGTGGGCAGGCGGATGCA
 L  R  N  Q  V  Y  L  E  D  G  L  D  P  F  P  G  E  G  R  V  V  G  R  Q  R  M  H

                   810       820       830       840       850       860       870       880
CAAGGCCTTGGACAGCGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTTCTGAACAGGCTCCCCAAGTTT
 K  A  L  D  R  V  E  E  H  P  G  S  R  M  T  A  E  K  F  L  N  R  L  P  K  F

                   890       900       910       920       930       940       950       960
TGATCCGGCAAGGCGAGGTGATTGACATCCGGGGCCCCATCAGGGACACCTTGCAGAACTGCTGCCCATTGCCTGCCCGG
 V  I  R  Q  G  E  V  I  D  I  R  G  P  I  R  D  T  L  Q  N  C  C  P  L  P  A  R

                   970       980       990      1000      1010      1020      1030      1040
```

FIG. 5

```
ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGCTGAGCGAGGAGAGAGGAGCCAGGAGTCACCCAACACCGGCACC
 I  Q  E  I  V  V  E  T  P  T  L  A  A  E  R  E  R  S  Q  E  S  P  N  T  P  A  P

    1050      1060      1070      1080      1090      1100      1110      1120
CCCGCTCTCCATGCTGCGCATCAAGTCTGAGAATGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACAACACCATTG
 P  L  S  M  L  R  I  K  S  E  N  G  E  Q  A  F  L  L  M  M  Q  P  D  N  T  I

    1130      1140      1150      1160      1170      1180      1190      1200
GGGACGTGCGAGCTCTGCTAGGCAGGCCAGGGTCATGGATGCCTCTGCCTTTGAGATCTTCAGCACATTCCCGCCCACC
 G  D  V  R  A  L  L  A  Q  A  R  V  M  D  A  S  A  F  E  I  F  S  T  F  P  P  T

    1210      1220      1230      1240      1250      1260      1270      1280
CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGGCTGCAGGCTGTGCCCAAAGCACTGCTGCTGCGGGCCACGCCGAGC
 L  Y  Q  D  D  T  L  T  L  Q  A  A  G  L  V  P  K  A  A  L  L  L  R  A  R  R  A
                                                                          t
                                                                          V

    COA-1a                ↓
    1290      1300      1310      1320      1330      1340      1350      1360
CCCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCTGTCCCGGTCCCGGTCCCGGTCCCAGTCCCGGTCCCGGTCCCGGCT
 P  K  S  S  L  K  F  S  P  G  P  C  P  G  P  G  P  G  P  S  P  G  P  G  P  G

    1370      1380      1390      1400      1410
CCAGTCCCTGTCCCGGACCCAGTCCCAGCCCCAATAAAGCACCCACCCCCTC
 S  S  P  C  P  P  G  P  S  P  S  P  Q
```

Sequenza del gene COA-1 con traduzione della proteina

b) Sequenza della sequenza accorciata del gene COA-1 usata per le trasfezioni

```
300       310       320       330       340       350       360       370       380       390       400
CCTCAGTGACTATGGCCTGCAGTGGGTGGGCGAGCCCATGGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGGACTGGATGACAGCCA
 L  S  D  Y  G  L  Q  W  V  G  E  P  M  D  Q  E  D  S  E  S  K  T  V  S  E  H  G  E  R  D  W  M  T  A

410       420       430       440       450       460       470       480
AGAAGTTCTGGAAGCCAGGGGGACTCATTGGGCGCCCCCTGAGGTGGACTTTGACAGGCTGCTGGCCAGCCTGCAGGATCTT
 K  K  F  W  K  P  G  D  S  L  A  P  P  E  V  D  F  D  R  L  L  A  S  L  Q  D  L

490       500       510       520       530       540       550       560
AGTGAGCTGGTGGTAGAGGGTGACACACCAGTGACACCAGTGCCCGGCGGGGCACGGCTGCGTACCCTCGAGCCCATCCC
 S  E  L  V  V  E  G  D  T  Q  V  T  P  V  P  G  G  A  R  L  R  T  L  E  P  I  P

570       580       590       600       610       620       630       640
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCCTCCACACAGCCTGCC
 L  K  L  Y  R  N  G  I  M  M  F  D  G  P  F  Q  P  P  Y  D  P  S  T  Q  R  C

650       660       670       680       690       700       710       720
TCCGAGACATATTGGATGGCTTCTTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGAC
 L  R  D  I  L  D  G  F  F  P  S  E  L  Q  R  L  Y  P  N  G  V  P  F  K  V  S  D

730       740       750       760       770       780       790       800
TTGCGCAATCAGGTCTACCTGGAGGATGGACTGGACCCCTTCCCAGGCGAGGGCCGTGTGGTGGGCAGGCAGCGGATGCA
 L  R  N  Q  V  Y  L  E  D  G  L  D  P  P  P  G  E  G  R  V  V  G  R  Q  R  M  H

810       820       830       840       850       860       870       880
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTTCTGAACAGGCTCCCCAAGTTT
 K  A  L  D  R  V  E  E  H  P  G  S  R  M  T  A  E  K  F  L  N  R  L  P  K  F

890       900       910       920       930       940       950       960
TGATCCGGCAAGGCGAGTGATTGACATCCGGGGCCCCATCAGGGACACCTTGCAGAACTGCTGCCCATTGCCTGCCCGG
 V  I  R  Q  G  E  V  I  D  I  R  G  P  I  R  D  T  L  Q  N  C  C  P  L  P  A  R

970       980       990       1000      1010      1020      1030      1040
ATCCAGGAGATTGTGGTGGAGACGCCCCACCTTGGCCGCTGAGGCGGAGAGGAGCCAGGAGTCACCGACCACCGGCACC
 I  Q  E  I  V  V  E  T  P  T  L  A  A  E  R  R  S  Q  E  S  P  N  T  P  A  P

1050      1060      1070      1080      1090      1100      1110      1120
CCCGGCTCTCCATGCTCGCGCATCAAGTCTGAGAATGGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACAACACCATTG
 P  L  S  M  L  R  I  K  S  E  N  G  E  Q  A  F  L  L  M  M  Q  P  D  N  T  I

1130      1140      1150      1160      1170      1180      1190      1200
GGGACGTGTCTGCTAGCCAGGCTCTGGCGCAGGCGGTCATGATGGATGCCTCGGCCTTTGAGATCTTCAGCACATTCCCGCCCACC
 G  D  V  R  A  L  L  A  Q  A  R  V  M  D  A  S  A  F  E  I  F  S  T  F  P  P  T

1210      1220      1230      1240      1250      1260      1270      1280
CTCTACCAGGACGACACTCTACGCTGCAGGCTGCAGCCTGTGCCCAAAGCAGCACTGCTGCTGCGGGGCACGCCCGAGC
 L  Y  Q  D  D  T  L  T  L  Q  A  A  G  L  V  P  K  A  A  L  L  R  A  R  R  A
```

```
      1290       1300       1310       1320
CCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCCTGTCCCGGTCC
 P   K   S   S   L   K   F   S   P   G   P   C   P   G   P
```
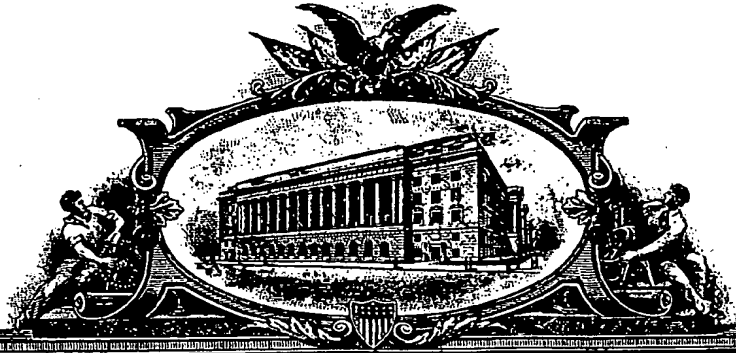
v

La sequenza sottolineata corrisponde al peptide immunogenico che abbiamo isolato. La freccia indica la sostituzione di base (c →t) e la conseguente sostituzione aminoacidica trovata nella sequenza isolata dalle cellule normali.

CGCTGCGGGACGGCTAGCGGCCCTGCGTGGAGGGCGAGGAATCCGCATCTATGGAGATGTCCCTGCATCCCATGACTCG
GA

GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAA
GG

ATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGACCCTCCGGCCGAGGCAACCTGCAGGCGCAGG
AG

GAACTGGAGACGATGTGTGTGCAGCTGCAGCGGCAGGTCAGGGGCAGGTCAGGGAGAGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAG
TG

GGTGGGCGAGCCCATGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGGACTGGATGACAG
CCA

AGAAGTTCTGGAAGCCAGGGGACTCATTGGCGCCCCTGAGGTGACTTTGACAGCTGCTGGCCAGCCTGCAGGATC
TT

AGTGAGCTGGTGGTAGAGGGTGACACCCAAGTGACACCCAGTGCCCGGGCGGGGCACGGCTGCGTACCCTGGAGCCCATC
CC

GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCTCCACACAGCGGCTGC
C

TCCGAGACATATTGGATGGCTTCTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCTTTAAGGTGAGTGA
C

TTGCGCAATCAGGTCTACCTGGAGGATGGACTGGACCCCTTCCCAGGCGAGGGCCGTGTGGTGGGCAGGCAGGGGATG
CA

CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTCTGAACAGGCTCCCCAAGTT
TG

TGATCCGGCAAGGCGAGGTGATTGACATCCGGGCCCCATCAGGGACACCTTGCAGAACTGCTGCCATTGCCTGCCC
GG

ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGCTGAGCGAGCAGGAGAGCCAGGAGTCACCCAACACACCGGC
ACC

CCCGCTCTCCATGCTGCGCATCAAGTCTGAGAATGGGAACAGGCCTTCCTACTGATGATGCAGCTGACAACACCATT
G

GGGACGTGCGAGCTCTGCTAGCGCCAGGGTCATGATGCCTCTGCCTTTGAGATCTTCAGCACATTCCGCCCAC
C

CTCTACCAGGACGATACACTCACGTGCAGGCTGCAGCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGCACGCCGA
GC

CCCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCCTGTCCCGTCCCGGCCCCAGTCCCGGCCCCGGTCCCGGTCCCGGC
T
CCAGTCCCGTCCCGGACCCAGTCCCAGCCCCCAATAAAGCACCCACCCCTC

PA 1287074

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APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/512,040*
FILING DATE: *October 15, 2003*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

PROVISIONAL APPLICATION FOR PATENT COVER SHEET
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)

Additional inventors are being named on the __0__ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

COLORECTAL ANTIGEN

CORRESPONDENCE ADDRESS

Direct all correspondence to:

[X] Customer Number: 26161

OR

[ ] Firm or Individual Name	
Address	
Address	
City	State		ZIP	
Country	United States	Telephone		Fax	

ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification	Number of Pages	36	[] CD(s), Number	
[X] Drawing(s)	Number of Sheets	14	[] Other (specify)	

[] Application Data Sheet. See 37 CFR 1.76.

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant Claims small entity status. See 37 CFR 1.27.

[X] A check or money order is enclosed to cover the filing fees.

[] The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 06-1050

[] Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($) $80

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Signature _____

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PROVISIONAL APPLICATION FOR PATENT

under

37 CFR §1.53(c)

TITLE: COLORECTAL ANTIGEN

APPLICANT:

M&C Folio: GBP88367

COLORECTAL ANTIGEN

The present invention relates to a novel, diagnostic antigen for colorectal cancer, uses thereof, and especially the use thereof in immunotherapeutic treatments for colorectal cancer.

Colon cancer is a leading cause of mortality in Western countries. Despite the improvement of surgery and chemotherapy treatments, the five-year survival rate has not significantly altered over several decades (1, 2). Immunological therapies have been intensively investigated in patients with melanoma, where treatment with IL-2, as well as the adoptive transfer of in-vitro cultured tumour infiltrating lymphocytes (TIL), has been found to result in cancer regression in a significant percentage of patients (3, 4).

In contrast, immunotherapy has not provided a benefit to colorectal cancer patients, which may be due to the poor immunological characterization of this cancer, limiting the treatment options for patients with this disease (5, 6). The presence of a $CD8^+$ T cell infiltrate in colon cancer has prognostic value (7); nevertheless, the presence of an inflammatory infiltrate was not linked to systemic immunity against cancer in this report. The loss of HLA class I expression both *in vitro* and *in vivo* has frequently been described in colorectal cancers, and appears to be associated with tumour progression (8-10).

The limited availability of in-vitro established tumour lines and specific T lymphocytes has in addition hindered analysis of the role of the immune system in colorectal cancer. Although a large number of tumour associated antigens (TAA) have been identified, the majority of these are either limited in their expression to melanoma or are expressed in melanoma as well as in a number of other histologies, including breast, ovarian, lung and prostate tumours (11).

Candidate antigens that appear to be over-expressed in colon cancer such as carcinoembryonic antigen (CEA), the epithelial cell adhesion molecule EP-CAM, HER-

2/neu and cyclophilin B, have been evaluated as potential targets for colorectal cancer therapy by carrying out *in vitro* sensitizations of PBMC with candidate peptides from these molecules that bind to particular HLA alleles.

However, a relatively small number of potential epitopes have been identified using this approach and the T cells that have been generated, using many of these peptides, did not efficiently recognise native, unmanipulated tumour cells (12-15).

We have now identified a new tumour associated antigen for colorectal cancer that is capable of eliciting a T cell-mediated immune response.

Thus, in a first aspect, the present invention provides a method for stimulating immunity against colorectal cancer, comprising stimulating the production of antibodies against the human homologue of the Socius gene product, wherein the alanine residue at position 399 is substituted by a valine residue. The coding sequence and the transcript thereof for the homologue are preferably as shown in Figure 5, which shows the relationship of genetic sequence with the COA-1 transcript.

What is particularly surprising is that it has been established that an epitope located between amino acids 371 and 384, inclusive, of the COA-1 transcript is responsible for stimulating immunity against the tumour variant of the protein, and that it is not necessary for the immunising peptide to comprise the mutation at position 399.

The immunising peptide comprises an epitopic portion of the peptide TLYQDDTLTLQAAG. This sequence may be supplemented with additional sequences at either end, up to and including the entire remaining sequences of COA-1, and even additional sequences beyond that, if desired, such as might be encountered with a fusion protein, for example. As demonstrated herein, more specific supplemental sequences, including FSTFPP at the N-terminus and/or LVPKAA at the C-terminus both permit stimulation. It will be appreciated that, in general, an epitope need not be as long as 14 amino acids, and that a deletion of a few amino acid residues from either end of the epitope should still serve to produce immunity.

It has also been established that the epitope is preferentially expressed by antigen presenting cells in association with the alleles HLA DRβ1*0402 or HLA

DRβ1*1301. It will be appreciated that these sympathetic alleles are not necessarily the only HLA alleles able to stimulate immunity to COA-1, and that the present invention extends to other sympathetic alleles.

Sympathetic HLA-II alleles are not necessarily present in all members of the human population but, where an individual has PBMC's (peripheral blood mononuclear cells) either autologous or allogeneic for either of these alleles, then it is sufficient simply to provide a vaccine comprising the immunising part of COA-1.

The immunising portion of COA-1 may be as much as the entire molecule, either with or without the mutation at position 399 but, more preferably, it simply comprises a peptide comprising at least the immunising epitope located between position 371 and 384 of the COA-1 transcript.

The immunising epitope may be presented in any suitable form. At its simplest, a vaccine comprising the peptide and a suitable carrier may be provided, together with, if required, any suitable excipients and/or adjuvants, for example.

The immunogenic peptide may also be presented in the form of nucleic acid in a form suitable for expression in the patient, either in a host organism, such as an attenuated virus, in a vaccine, or in the form of a suitable expression vector for expression *in vivo*.

It will be appreciated that the present invention extends to the sequence for COA-1, as well as the transcription product thereof. The invention further extends to the COA-1 sequence lacking one or more introns. The sequence of the invention may also lack one or more exons, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded. It is not necessary for the amino acid substitution at position 399 to be encoded, and it is generally preferred that this substitution is not encoded by the nucleotide sequences of the present invention. Without being bound by theory, it is possible that this substitution in the sequence of normal cells could affect the processing of the antigen, leading to a lack of expression of the immunogenic epitope. It will be appreciated that the degeneracy of the genetic code allows the nucleotide sequence to vary widely and still encode the

immunogenic sequence, but it is generally preferred to use the wild-type sequence, for simplicity, unless it is desired to engineer a splice site, for example.

Where the patient does not express a sympathetic HLA-II allele, then immunity may be conferred in a number of ways, any of which may also be employed in patients expressing a sympathetic allele.

Sympathetic alleles are expressed by PBMC's, such as B cells and fibroblasts. Thus, in one aspect, it is sufficient to isolate PBMC's or their progenitors from the patient and to transform these cells with HLA DRβ1*1301 or HLA DRβ1*0402 alleles, for example. Once successful transformation has been achieved, then the PBMC's, whether directly transformed, or whether obtained from the progenitors, may be used to stimulate the appropriate immunity, after reintroduction into the patient. This may be achieved either by introducing the PBMC's into the patient, followed by administration of a vaccine as described above, or the PBMC's may be contacted with COA-1, or a precursor therefor, or the immunising epitope or precursor therefor and, preferably once there has been some opportunity for endocytosis to occur, the treated PBMC's are administered to the patient. It will be appreciated that, in these circumstances, a "precursor" may include, for example, a fusion protein or a nucleic acid suitable for expression in the PBMC culture.

It will also be appreciated that suitable PBMC's may be obtained from, for example, a universal donor, and an immunising preparation may be made from such cells in a manner similar to that described above for transformed cells from the patients themselves.

It will be appreciated that the present invention extends to vaccines and immunising preparations as described above, as well as to host cells expressing COA-1, or a precursor therefor, provided that the immunising epitope is comprised in the transcript expressed thereby.

It will also be appreciated that the present invention extends to the use of antibodies recognising COA-1 mutated at position 399. Such antibodies may be used as a passive vaccine, for example or may be used in diagnostic assays for colorectal

cancer. Such assays may take the form of ELISA assays, for example, or may be used in suitable immunoblotting techniques.

Figure 1 shows a phenotypic characterization of the colorectal cancer line 1869 col.

Figure 1A shows a stained 1869 col cell line using antibodies directed against MHC class I (W6/32) and class II (L243) molecules, an epithelium marker (Ber-EP4), and the β subunit of prolyl-4-hydroxylase (5B5), a protein expressed exclusively in fibroblasts.

Figure 1B shows intracellular staining carried out using three cytokeratin reactive monoclonal antibodies: CK18, which reacts with cytokeratin 18; LP34, which reacts with multiple cytokeratins; and MNF116, which reacts with cytokeratins 5, 6, 8, 17 and probably 19.

Figure 1C shows staining of 1869 col cells at passage 6 (P6) and passage 20 (P20), carried out with the anti-CEA monoclonal antibody Col-1.

Figure 2 shows a cDNA clone isolated from the 1869 cDNA library encoding an antigen recognised by C111 T cells. The 293 cells expressing the MHC DRβ1*0402 or 1301 molecules were transfected with the 1D8 cDNA clone, or COA-1a, which corresponds to nucleotides 209-1318 of the COA-1 gene (see Figure 3). Target cells were either transfected with the COA-1a product alone or were co-transfected with a mixture of COA-1a and the full length HLA class II invariant chain (Ii). Additional targets were transfected with a control plasmid encoding GFP. Eighteen hours following the addition of 5x10⁴ C111 T cells to the transfectants, supernatants were collected and IFN-γ release was measured by ELISA.

Figure 3 provides the sequence of the COA-1 gene isolated from the mRNA of the tumour line 1869 col. The COA-1 gene was isolated by RT-PCR from the 1869 col tumour cell line. The sequence of the 1D8 cDNA clone is shown in bold letters. The amino acid sequence corresponding to the T cell epitope is underlined, and the single nucleotide difference between the normal and tumour transcripts at position 1280 is noted. The amino acid sequence of the longest open reading frame in this transcript,

which is similar to the Socius gene product (20), is noted beneath the nucleotide sequence.

Figure 4 shows that the COA-1 transcript derived from normal B cells is not recognised by the clone C111 T cells. 293 cells expressing the indicated MHC DRβ1 molecules were transfected with COA-1a cDNAs isolated by RT-PCR from either the 1869 col cell line or from 1869 CD40L stimulated B cells. The GFP and Ii-1D8 constructs were used as negative and positive controls, respectively. Eighteen hours following the addition of 5×10^4 C111 T cells to the transfectants, supernatants were collected and IFN-γ release was measured by ELISA.

Figure 5 shows the relationship of the genetic sequence of COA-1 to the transcript, and the position of the primers used to generate the COA-1a product is shown by the arrows.

Several tumour reactive CD4$^+$ T lymphocytes were isolated from PBMC and TIL that were obtained following the establishment of autologous cultured colon tumour cell lines. These studies focused on a single clone of CD4$^+$ T cells, C111, that responded strongly to autologous tumour cells, and demonstrated low but significant reactivity with autologous EBV B cells, but failed to respond to autologous CD40L stimulated B cells. The gene encoding this antigen, termed COA-1, was isolated by screening an autologous cDNA library with clone C111 T cells. This gene appeared to be nearly identical to the gene encoding the human homologue of the rat Socius protein that was recently cloned using a yeast two-hybrid screening assay in which a member of the Rnd family of GTPases was used as bait (20). The Socius product was expressed at high levels in rat testis, but was expressed at significantly lower levels in rat lung, thymus and brain.

The longest open reading frame in the COA-1 transcript encodes a 437 amino acid product that corresponds to a portion of the human Socius gene product, and two overlapping peptides derived from this open reading frame were identified that could sensitise target cells expressing either HLA-DRβ1*0402 or 1301. The stimulation

observed with peptide pulsed targets was weak relative to that seen with the tumour cell lines that were recognised, and a minimum concentration of approximately 10 μM was needed to stimulate significant cytokine release from C111 T cells (Table 4).

Peptides derived from non-mutated tumour antigens such as tyrosinase (23) and TRP-1 or TRP-2 (17) have also been found to stimulate only relatively low levels of cytokine release from HLA class II-restricted, tumour reactive T cells, and minimal concentrations of between 1 and 10 μM of the peptides identified in these studies were required to sensitise target cells for T cell recognition. This may reflect the fact that these represent non-mutated self antigens, and that self tolerance results in the deletion of T cells that recognise peptides that bind to class II molecules with high affinity.

In addition, the autologous tumour cell line should present this peptide in the context of both the HLA-DRβ1*0402 and 1301 restriction elements, leading to enhanced stimulation of T cells reactive with this epitope. Transfectants expressing the COA-1 product stimulated significantly less cytokine release from C111 T cells than the autologous tumour cell line that had been induced to express high levels of HLA class II molecules. One potential explanation for this observation, however, is that the HLA class II positive 293 cells used as targets for transfection of the COA-1 gene products fail to express optimal levels of accessory molecules associated with the processing of this epitope.

The COA-1 transcript is nearly identical to sequences derived from a variety of tissues and tumour cell lines. These transcripts, however, comprise a large array of over 20 alternatively spliced products that are derived from at least 15 exons residing at the chromosome 1p36.1-p35 locus. The COA-1 product expressed in colon tumour cell lines appeared to contain a unique splicing pattern that did not correspond to any of the transcripts identified in the EST and GenBank databases, which may not encode products recognised by C111 T cells. Two nearly identical COA-1 gene products were amplified from EBV B cells, one of which was identical to that isolated from the colon tumour cells, and a second that contained a single nucleotide alteration at position 1280 that resulted in a substitution of a valine residue for the alanine residue at position 399

encoded by the dominant colon tumour cell product. It is not clear why C111 T cells only appeared to weakly recognise EBV B cells expressing the appropriate HLA class II gene products, but these observations could result from inherent differences in the antigen processing abilities of colon tumour cells and EBV transformed B cells.

Previous results have suggested that differences in the proteosomal subunits expressed by various cells may significantly influence antigen recognition, which provides one potential explanation for this finding (24). The RT-PCR products that were amplified from normal B cells and fibroblasts also appeared to uniquely encode the COA-1 variant that expressed a valine residue at amino acid 399, and target cells that were transfected with the COA-1 product that was amplified from normal cell lines were not recognised by C111 T cells.

Thus, it appears that normal B cells and fibroblasts either fail to express the COA-1 transcript that can be processed and presented to C111 T cells or express this product at only relatively low levels. The mechanisms involved in the preferential expression of these two transcripts are unknown, but these may represent the products of two nearly identical genes whose expression is differentially regulated. The correlation between expression of these products and the ability of C111 T cells to recognise the epitope encoded by these products provides further evidence that this represents the natural product recognised by these T cells and not a peptide mimic of the natural epitope.

An additional observation that is unexplained is how the alteration at position 399 affects recognition of the cell epitope comprised of amino acids 371 to 384 of the COA-1 transcript. Results of a previous study indicated that alteration of a distal residue can influence the ability of tumour reactive CD4+ T cells to recognise a mutated product of the CDC-27 gene product (21). Preliminary results presented in the prior study indicated that altered intracellular targeting of the mutated CDC-27 gene product may have played an important role in influencing processing of this gene product. Investigation of the cellular localization of the COA-1 protein in normal and tumour cells may help to indicate whether a similar mechanism may be involved with T cell recognition of this product.

Transfection studies, as well as peptide pulsing experiments indicated that either of the autologous HLA-DRβ1 alleles, DRβ1*0402 or DRβ1*1301 could present the T cell epitope to clone C111 T cells, which may potentially enhance the immunogenicity of this peptide in patient 1869 as well as other individuals that express these class II alleles. This observation is not unique, however, as examples of promiscuous recognition of class II and well as class I restricted epitopes have been noted in previous studies. In one report, CD4+ T cells were identified that also recognised an epitope of the herpes simplex type 2 virus virion protein, VP16 in the context of DRβ1*0402, 1102 or 1301 but not several closely related DR4, 11 or 13 subtypes (25). The sequences of the DRβ1*0402, 1102 and 1301 molecules are identical in a polymorphic region between amino acids 67 and 71, and site directed mutagenesis studies demonstrated that these residues were critical for the recognition of the viral epitope.

High levels of lymphocyte infiltration into tumours has been shown in some studies to be correlated with a good prognosis (26), but detailed investigations of the reactivity of infiltrating T cells have not been carried out. The expression of HLA class II molecules on colorectal cancer cells is also a favourable prognostic marker (27) (28). Previous studies resulted in the isolation of HLA class I (29) and class II (30, 31) restricted tumour reactive T cells from colon cancer patients, but only a limited panel of shared tumour specific antigens were identified in these studies. This report presents the description of an immunogenic CD4+ T cell epitope derived from a previously un-described colorectal cancer antigen that represents a potential target for immunotherapy in patients with this disease. Evaluation of the in vitro immunogenicity of the COA-1 epitope in DRβ1*0402 and *1301 positive colorectal patients may provide support for the use of this antigen in the immunotherapy of patients with this disease.

Example

Material and Methods

Cell lines and antibodies.

Colon cancer lines were generated from tumour liver metastases of five patients admitted to the Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. The cell lines were generated from the tumour samples by cutting the tissue into small fragments, followed by filtration through sterile gauze. The tumour cells were cultured in collagen-coated 6-well plates (Becton Dickinson, Franklin Lakes, NJ) in ACL-4 medium (InVitrogen, Carlsbard, CA) containing 10 % foetal bovine serum plus MEGM SingleQuots (Clonetics, Walkersville, MD) that contained epidermal growth factor (10 ng /ml), insulin (5 μg/ml), hydrocortisone (0.5 μg /ml), gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml). Fresh medium was added to the cells every 5 days and fibroblasts were depleted from the cultures by carrying out a short-term treatment with trypsin. Immunofluorescent staining assays to assess cell surface HLA gene expression were carried out using the anti-class I mAb W6/32 and the anti-DR mAb L243 (Becton Dickinson).

The cell lines were stained using the mAb BerEP4 (DAKO, Cupertino, CA) that is directed against a cell surface molecule whose expression appears to be limited to epithelial tissues, and intracellular staining was carried out using the cytokeratin reactive mAbs CD18, LP34 and MNF116 (DAKO). Analysis of the expression of carcinoembryonic antigen (CEA), a molecule that is frequently over-expressed in colon tumour, was carried out using the mAb Col-1 (Zymed, South San Francisco, CA). The presence of fibroblasts in the cultured colon tumour cell lines was assessed using the mAb 5B5 (DAKO) that was directed against the β subunit of prolyl-4-hydroxylase, a protein involved with the synthesis of collagen. Flow cytometry was carried out using a FACScan (Becton Dickinson). The established colon cancer lines SW1463, SW480 and Colo205 were obtain from American Type Culture Collection (ATCC, Manassas, Virginia). The melanoma cell line 1681, the fibroblast cell line 1519 and the EBV-transformed B cell lines 1869 and 1519 were established in the Surgery Branch and

were cultured in RPMI plus 10 % FBS. The normal B cell lines 1847, 1681, 1872 and 1869 were generated, as previously described (16), by culturing PBL in ISCOVE's medium (InVitrogen) plus 10 % human serum in the presence of 100 IU/ml of CD40L (Immunex, Seattle, WA) and 100 IU/ml of recombinant human IL-4 (Pharmingen, San Diego, CA). The MHC class I and class II typing of the PBL and of the tumour lines used in this study was determined by single-stranded oligonucleotide probe–PCR typing carried out in the NIH HLA typing laboratory, and is summarised in Table 1. Antibodies used to carry out T cell receptor (TCR) analysis were obtained from Beckman/Coulter (Miami, FL) or Pierce/Endogen (Rockford, Il.).

Identification and characterization of tumour reactive T cells.

Tumour reactive T lymphocytes were generated from PBMC and tumour infiltrating lymphocytes (TIL) derived from colon cancer patients. Incubation of PBMC with autologous tumour cells that had been irradiated with 150 Gy was carried out at a tumour cell to lymphocyte ratio of 1 to 5 in RPMI media containing 300 IU/ml of recombinant human IL-2 plus 10 % human serum (HS). The cultures were stimulated weekly for a period of 5 to 6 weeks with autologous irradiated tumour cells. Cultures of TIL were established by initially plating fresh uncultured tumours at 5×10^5 cells per well in 24-well plates in RPMI containing 10 % HS and 1,000 IU/ml of IL-2. Tumour cells used for T cell stimulation were cultured for at least 10 days in RPMI containing 10 % HS to avoid the generation of T cells with reactivity against FBS. In addition, to optimise or up-regulate the expression of MHC molecules by tumour cells, these cells were incubated with IFN-γ (500 IU/ml) for 48 hr. The reactivity of the T cell lines against colon cancer lines was examined by incubation of 2×10^4 or, for some of the assays, 5×10^4 T cells in flat bottom 96-well plate in the presence of 5×10^4 autologous or allogeneic tumour cells. After overnight incubation at 37°C in 5 % CO_2, the supernatants were collected and T cell responses were evaluated using anti-IFN-γ antibodies (Endogen, Rockford, IL) in a sandwich ELISA assay.

After 3 weeks of culture the T cell lines were cloned by limiting dilution in the presence of allogeneic PBMC that had been irradiated with 50 Gy in RPMI media containing 30 ng/ml of OKT3 mAb in RPMI plus 10 % HS. The following day, fresh medium plus rh-IL-2 (300 IU/ml) was added to the cultures. After two weeks of culture, growth positive wells were screened for their ability to release IFN-γ in response to tumour stimulation. The T lymphocytes from sensitised PBMC that were chosen for further analysis, C4, C49 and C111, were isolated from cultures that were plated at 5 cells per well, but only 27% of the wells were positive for growth under these conditions, showing that some or all of these cells represent T cell clones.

Analysis carried out with antibodies directed against T cell receptor (TCR) families showed that greater than 95% of clone C4 T cells expressed a TCR reactive with an anti-Vβ5 reactive antibody, whereas C49 failed to express TCRs detected by any of the commercial antibodies. Amplification of the clone C111 TCR Vβ region product carried out using RT-PCR showed that this clone expressed a single sequence derived from the Vβ18 germline gene. Flow cytofluorimetric analysis showed that approximately 80% of C111 T cells expressed Vb18, but contaminating feeder cells used to expand the T cell clone may be responsible for the discrepancy between these results. Two CD4+ tumour reactive T cell cultures, C5 and C15, were also identified from 1869 TIL. These cultures were isolated from cells that were plated at one cell per well, and, as only 3% of the wells that were plated were positive for growth, these represent T cell clones. In addition, these cultures stained homogeneously with an antibody directed against Vb2, further showing that these represented T cell clones.

Tumour reactive cultures were then expanded in the presence of allogeneic PBL that were irradiated with 50 Gy in RPMI containing PHA (1µg/ml) and IL-2 (300 IU/ml). Immunofluorescent analysis of positive cultures was carried out using mAb directed against CD3, CD4, CD8, CD16, and CD56 (Becton Dickinson). Antibody blocking assays were carried out by pre-incubating target cells for 1 hour with W6/32, an antibody directed against a pan-MHC class I epitope, or L243, a mAb directed against a pan-HLA class II DR epitope. The T cells were then added to target cells, and IFN-γ release measured following an overnight incubation.

CIITA transduction of tumour lines.

In order to induce stable expression of cell surface MHC class II molecules, the tumour lines 1869 col, SW480, and Colo205 were transduced with a recombinant retrovirus that was generated by cloning the gene that encoded the human class II transactivator (CIITA) into the retroviral expression vector pCLRCX (17). The transduced 1869 tumour cells were then sorted using a FACSVantage[TM] cell sorter (Becton Dickinson) to obtain cells that homogeneously expressed relatively high levels of cell surface HLA class II expression.

Isolation of MHC class II DRβ1 molecules.

The DRβ1*0402 gene was isolated by carrying out an RT-PCR with RNA derived from the tumour line 1869 col, and the DRβ1*1301 gene was obtained by carrying out an RT-PCR with RNA derived from an autologous T cell line. Primers that were used to amplify HLA-DR were: 5'-TCCAGCATGGTGTGTCTGA-3' and 5'-CCTTGAATGTGGTCATCT-3'. Two additional primers were designed to specifically amplify the HLA-DR13 gene product: 5'CGTTTCTTGGAGTACTCTACGTC-3' and 5'-CCACCGCGGCCCGCTCGTCT-3'. The isolated products were cloned in the plasmid vector pCR-Blunt (Invitrogen, Carlsbard, CA) and sequenced using an ABI Prism 310 Genetic analyser (Perkin-Elmer, Shelton, CT). The genes were then cloned in the eukaryotic expression vectors pCDNA3.1 (Invitrogen) and the retroviral expression vector CLRCX4, discussed above.

Constructs encoding either of the HLA-DRβ1 genes were co-transfected along with a construct encoding the HLA-DRα gene into 293 cells. Stable transfectants were stained with the FITC labelled anti-HLA-DR mAb L243, and cells that were strongly positive for the expression of the cell surface HLA-DR molecules were isolated using a FACSVantage[TM] cell sorter (Becton Dickinson). To induce the expression of molecules involved with HLA class II antigen processing, such as the class II invariant chain,

MB genes, the 293 cells that had been transfected with the HLA-DR

re then transduced with recombinant retroviral supernatants generated

C-CIITA construct, as previously described (17).

Library Construction and screening.

tNA was extracted from 1869 col tumour line using Triazol (GIBCO,

y (A) RNA was then isolated using poly (A) Tract (Promega, Madison,

y (A) RNA was then converted to cDNA using the SuperScript cDNA

(InVitrogen) and cloned in the episomal mammalian expression vector

BioSystems, Gaithersburg, MD). The pEAK8 vector had been modified

ragment encoding amino acids one to 80 of the human invariant chain (Ii)

f the EF1-α promoter in order to express the cDNA inserts as fusion

target the gene products to the HLA class II antigen presentation

The recombinant cDNA was then electroporated into DH10B

ent cells (InVitrogen), and plasmid pools containing approximately 50

inants prepared as previously described (18). The 293 cell lines that were

th HLA-DRβ1*0402 (293-DR0402) or HLA-DRβ1*1301 (293-DR13)

ly transfected with DNA prepared from the cDNA pools (200 ng) using

2000 (InVitrogen) according to the manufacturer's directions.

r to conserve C111 T cells, screening assays were initially carried out by

mixture of $5x10^4$ 293-DR*0402 and $5x10^4$ 293-DR*1301 cells with

pools in 96 well flat bottom plates. The following day the cells were

10^5 cells T cells in AIM-V medium plus 2 % HS were added each well.

f incubation at 37^0C and 5 % CO_2, 100 µl of supernatant was collected

release was evaluated by ELISA. For subsequent assays, cDNA pools

re transfected into 293 cells that expressed only a single HLA DR allele,

were tested for their ability to stimulate C111 T cells.

5' Rapid amplification of cDNA ends (RACE).

Total RNA was extracted from the 1869 col tumour cell line and a 5' RACE was performed using the Smart RACE cDNA amplification kit according to the manufacturer's instructions (Clontech, Franklin Lakes, NJ). The RT-PCR products were cloned into the pCDNA 3.1 Topo cloning vector (Invitrogen) and recombinant DNA was prepared for sequence analysis. In addition, amplification of the full length COA-1 gene products was carried out using the Advantage 2 PCR kit (Clontech). The amplification was carried out by incubation at 95^0C for 1 minute, followed by 35 amplification cycles consisting of a 30 second incubation at 95^0C, a 30 second annealing step at 62^0C, and a 2 minute extension step at 68^0C.

Identification of T cell epitopes.

Peptides of 20 or 21 amino acids in length that overlapped by 15 amino acids that were encoded by the long open reading frame of the original cDNA clone that was isolated were synthesised by solid-phase method using a peptide synthesiser (AMS 422; Gilson Co., Inc. Middleton, WI). The purity of the peptides was verified by mass spectrometry (Tuft's Core Facility, Boston, MA). Allogeneic B cells ($1x10^5$ cells/well) that expressed either the DRβ1*0402 or the DRβ1*1301 molecules were incubated with 50 µg/ml in 100 µl/well of ISCOVE'S medium plus 10 % HS in flat bottom-96-well plates. After three hours, $1-5x10^4$ T cells were added to the wells in 150 µl/well of medium and incubated for 18 hours at 37^0C and 5% CO2, followed by measurement of INF-γ release by ELISA..

Results

Generation and characterization of colon cancer lines.

Cultured colon cancer lines were initially established from liver metastasis specimens obtained from five colorectal cancer patients. Analysis of one of the most rapidly proliferating cell lines that was obtained, 1869 col, demonstrated that these cells expressed a common epithelial marker, expressed cytokeratins associated with epithelial cells (Fig.1), and maintained a morphology in tissue culture that was typical of epithelial cells (data not shown).

In contrast, the cell lines did not stain with an antibody directed against the β subunit of prolyl-4-hydroxylase, a cell surface marker expressed in fibroblasts. Taken together, these results indicated that these cells were of epithelial origin and represented colon cancer cell lines and did not contain significant numbers of normal cells. The 1869 col cell line expressed uniform levels of MHC class I molecules and low or undetectable levels of cell surface MHC class II molecules were found on the same cells (Fig. 1), but treatment of the 1869 col cells with IFN-γ resulted in strong up-regulation of HLA class II expression (data not shown).

The carcinoembryonic antigen represents a marker that is expressed at high levels *in vivo* on colon tumour cells as well as on many colon tumour cell lines, but is not expressed by fibroblasts or hepatic cells. Analysis of 1869 col cells indicated that they expressed CEA (Fig. 1), and the additional colon tumour cell lines that were generated appeared to express similar levels of this gene product (data not shown). An early passage of the 1869 col cell line demonstrated high level expression of CEA, and lower but still significant levels of CEA expression were observed at later passages of 1869 col cells (Fig. 1). These observations are consistent with previous studies in which heterogeneous expression of CEA was observed on a variety of colon tumour cell lines (19).

Isolation and characterization of colon cancer reactive T lymphocytes.

In the initial attempts to derive colon tumour reactive T cells, tumour infiltrating lymphocytes (TIL) from patient 1869 were cultured in high dose IL-2. In addition, autologous tumour cells, that had been treated with IFN-γ to up-regulate HLA class II gene expression, were used to carry out in vitro mixed lymphocyte tumour cultures (MLTC) with PBMC from patient 1869. Three CD4⁺ tumour reactive T cell clones, C4, C49 and C111, were initially selected for further analysis on the basis of their high degree of reactivity with the autologous tumour cell line.

The three clones derived from PBMC released IFN-γ in response to autologous tumour cells that had been treated with IFN-γ, and these clones released significantly higher levels of IFN-γ in response to 1869 tumour cells that had been treated with the CIITA and sorted for cells that constitutively expressed high levels of cell surface HLA class II molecules (Table 2).

Relatively low levels of IFN-γ were released following stimulation with the autologous 1869 EBV B cell line from the three T cell clones. All of the T cell clones released IFN-γ and GM-CSF but not IL-4 following stimulation with HLA class II positive tumour cells (data not shown), indicating that they represent cells of the Th1 cell phenotype.

In order to test whether the clones isolated from the PBMC recognised tumour cells in an MHC-restricted manner, cytokine release assays were carried out in the presence of anti-HLA class I and class II specific antibodies using stimulator cells bearing a variety of MHC haplotypes (Table 1). The results indicated that the C4, C49 and C111 T cell clones recognised the autologous tumour cells in the context of the HLA DR class II restriction element (Table 2). The C49 and C111 T cell clones also recognised the CIITA transduced allogeneic MHC class II⁺ colon cancer lines SW480 and Colo 205 that shared expression of HLA-DRβ1*1301 with the autologous tumour, and this recognition was blocked by pre-incubation of the tumour cell lines with the anti-HLA-DR mAb.

Generally the responses were inhibited by between 50 and 90% by pre-incubation with the anti-HLA DR antibody, whereas less than 20% inhibition was observed with the anti-HLA class I antibody. The response of the C4 line to the SW480 CIITA treated tumour cell lines, as well as the response of C111 to theColo205 CIITA, were only partially inhibited by anti-HLA DR antibody, which might reflect the fact that these T cells can recognise additional ligands other than the classical TCR. The C4, C49 and C111 clones recognised autologous EBV B cells as well as an allogeneic EBV B cell line that shared expression of HLA DRβ1*1301 with autologous cells. Normal B cells that were generated by stimulating autologous PBMC with CD40 ligand plus IL-4, as well as an allogeneic fibroblast cell line that shared expression of HLA DRβ1*1301 with the 1869 col tumour and that was treated with IFN-γ to up-regulate HLA class II gene expression, stimulated little or no cytokine release from these T cells (Table 3).

Two CD4+ T cell clones from TIL 1869 that responded in preliminary assays to autologous HLA class II positive tumour cells were also tested for their ability to recognise autologous as well as allogeneic colon tumour cell lines. Clones C4, C49 and C111, as well as two clones derived from 1869 TIL, C5 and C15, responded to the allogeneic colon tumour cell line 1847 col that shared expression of the HLA-DRβ1*1301 gene product with the autologous tumour. In contrast, the allogeneic 1872 col cell line that did not share expression of any HLA DR gene products with the 1869 col tumour failed to stimulate significant cytokine release from the T cell clones.

Identification of the antigen recognised by C111 T cells.

Further studies aimed at identifying tumour antigens expressed on 1869 col cells focused on C111 T cells, which was the only T cell clone that expanded sufficiently to allow the cDNA library to be screened. The results of studies carried out with additional tumour histologies indicated that C111 T cells did not recognise two allogeneic renal cell lines, as well as a prostate tumour cell line that shared expression of HLA-DRβ1*1301 with the 1869 col cell line (data not shown). A single allogeneic

melanoma cell line that expressed HLA-DRβ1*0402 was identified, 1681 mel. Cell surface HLA class II expression was up-regulated following treatment of the 1681 mel cell line with IFN-γ, and the treated cells were recognised by C111 T cells, indicating that certain tumour types shared expression of the antigen recognised by these T cells (Table 3).

Stable transfectants of the 293 cell line that expressed either the autologous MHC class II DRβ1*0402 or 1301 gene products molecules were then mixed in equal numbers and transiently transfected with DNA pools generated from the autologous tumour cell cDNA library. The positive pool that was initially identified following the screening of approximately 3×10^4 clones, 4G3, appeared to sensitise either 293-DRβ1*0402 or 1301 target cells for recognition by C111 T cells, and a single cDNA clone that could sensitise target cells for recognition by C111 T cells, 1D8, was identified (Fig. 2).

An assay carried out by transfection of the 293-DRβ1*0402$^+$ or 1301$^+$ cell lines individually with the 1D8 cDNA indicated that either of these HLA class II restriction elements could present the T cell epitope to C111 T cells. In contrast, 293 cell lines that expressed the HLA-DRβ1*0101, 0401, 0701 or 1601 class II alleles failed to stimulate these T cells following transfection of the 1D8 cDNA clone (data not shown), indicating that presentation of this epitope to C111 T cells may be limited to the two autologous HLA-DR alleles expressed by 1869 col cells. Further screening of the cDNA library resulted in the isolation of a second cDNA clone that was nearly identical to the 1D8 clone. The isolation of a second clone with a nearly identical sequence supports the finding that this represents the natural transcript encoding the antigen recognised by C111 T cells

Characterization of colorectal tumour associated antigen COA-1.

The 1D8 insert contained a 44 bp polyA tail at the 3' end, but appeared to represent a partial cDNA clone as it was only 291 bp in length. The 5' end of the gene

product that was expressed in the 1869 col cell line was then isolated by carrying out a rapid amplification of cDNA ends (RACE) reaction using nested internal primers complementary to the sequence of the 1D8 clone. Sequencing of products that were cloned from this reaction indicated that a 1412 bp product represented the predominant transcript of the gene in the 1869 col cell line that encoded the antigen recognised by C111 T cells, which was designated colorectal antigen-1 (COA-1) (Fig 3).

Comparison of the COA-1 sequence with the genomic DNA sequence database indicated that this product was derived from 13 exons, but at least two additional alternatively spliced products of this gene were isolated from the RACE reaction. An alignment of the COA-1 transcript with the human EST database indicated that this was identical or nearly identical to several sequences obtained from normal human brain, placenta, ovary, and testis, as well as sequences obtained from a variety of adenocarcinomas.

The 5' end of the transcript cloned from the RACE reaction corresponded to the 5' end of several EST sequences found in the database, and the 3' end of the original cDNA clone corresponded to the 3' end of the EST transcripts derived from several cell lines, indicating that these may represent the authentic 5' and 3' ends of the predominant COA-1 colon tumour cell transcript. The COA-1 sequence was also nearly identical to that of a transcript encoding the human homologue of the rat Socius protein, a molecule that was recently cloned on the basis of its ability to bind to a member of the Rnd family of GTPases (20).

Forward and reverse primers located at or near the 5' and 3' ends of the putative COA-1 gene product were then used to carry out an RT-PCR from 1869 RNA, as the RACE products that had been cloned only comprised a portion of the normal transcript. When RT-PCR was carried out with several primers that were proximal to the putative 5' end of the transcript in combination with primers that were complementary to the highly repetitive G/C rich sequence near to the 3' end of the COA-1 transcript, a variety of non-specific transcripts were generated (data not shown). A product that was designated COA-1a was, however, successfully amplified from 1869 col RNA using

two primers that encompassed the region between nucleotides 290 and 1318 of the putative full length COA-1 transcript.

Transfectants that co-expressed the COA-1a gene along with either HLA-DRβ1*0402 or 1301, appeared to stimulate comparable levels of cytokine release from C111 T cells to those transfected with the truncated 1D8 cDNA clone, showing that the full length gene can be processed relatively efficiently (Fig. 2). Co-transfection of the COA-1a gene with a construct encoding the full length human invariant chain (Ii) had little or no effect on the recognition of target cells transfected with the COA-1a product by C111 T cells. Thus, either the levels of Ii expression in 293 cells that were also transfected with a construct encoding the CIITA gene product was adequate for recognition of this epitope, or Ii expression does not have a significant impact on the processing of the COA-1 epitope.

In addition, the COA-1a product was not fused with amino acids one to 80 of the human Ii molecule, which had previously been shown to enhance the recognition of some HLA class II antigens (21).

The observation that the fusion of the cDNA clone with the invariant chain did not enhance recognition by the CD4+ T cells shows that the COA-1 antigen may naturally target the endogenous HLA class II processing pathway in colon tumour cells.

The expression pattern of the COA-1 gene was then examined in several colorectal, melanoma, and EBV-B cell lines, as well as in several normal cell lines which included CD40L stimulated B cell and fibroblast cell lines. The results of Northern blot analysis indicated that this gene was expressed at relatively low levels in colon and melanoma tumour cell lines, EBV B cells, normal B cells and fibroblasts, and quantitative TaqMan RT-PCR indicated that the levels of expression did not differ significantly between these cells (data not shown).

The observation that the level of expression of the COA-1 gene did not differ significantly between cell lines that were or were not recognised by C111 T cells, showed that these cells express similar but non-identical products. Therefore,

transcripts of the COA-1 gene that were expressed in the autologous and allogeneic CD40L stimulated B cells, as well as allogeneic fibroblast cell lines, were isolated using RT-PCR and sequenced.

The results of sequencing carried out with the bulk RT-PCR products showed that CD40L stimulated B cells and fibroblast cell lines predominantly expressed products that appeared to be identical to the COA-1 transcript derived from 1869 col cells with the exception of a single substitution of a T for a C residue at nucleotide position 1280, resulting in a change at amino acid 399.

The COA-1 transcripts that were expressed in CD40L B cells were isolated by carrying out RT-PCR and cloning the resultant products. Ten out of ten clones from the CD40L B cells that were sequenced contained a T at position 1280 but were otherwise identical to the 1869 col COA-1 transcript.

Amplification of the COA-1 gene product from allogeneic colorectal tumour lines SW1463, SW480 and 1847 col, as well as the 1681 mel line, showed that these cells predominantly expressed products containing a C residue at position 1280, as determined by sequencing the bulk, un-cloned RT-PCR products that were amplified from these cells (data not shown). Two peaks of comparable heights that corresponded to C and T residues at position 1280 of the COA-1 transcript were derived by sequencing the un-cloned RT-PCR product from autologous EBV B cells, indicating that these products may be expressed at similar levels in these cells. The results obtained using RNA from autologous CD40L stimulated B cells, EBV B cells, and the colon tumour cell lines were confirmed by repeated analysis carried out on products obtained from four independent RT-PCR reactions, showing that the residue found at nucleotide 1280 of the COA-1 transcripts did not represent a PCR mutation (data not shown).

To evaluate the significance of the single base pair change at position 1280 in the COA-1a sequence, the RT-PCR products obtained from autologous CD40L stimulated B cells were cloned in a eukaryotic expression vector. A plasmid containing the COA-1a transcript that was amplified from the normal B cells was then compared

with products cloned from 1869 col cells for its ability to sensitise 293-DR*0402 or 293-DR*1301 cells for recognition by C111 T cells. Target cells expressing either of the autologous HLA-DR genes that were transfected with the COA-1a or 1D8 gene products, but not the product that was isolated from CD40L activated B cells, stimulated cytokine release from C111 T cells (Fig. 4). These results showed that there was a correlation between the recognition of normal B cells and tumour cells and the ability of the COA-1 gene products that were expressed by these cells to sensitise targets for recognition by C111 T cells.

Identification of the epitope recognised by the CD4^{+} clone C111.

The results of transfection studies carried out using truncated COA-1 gene products showed that the C111 T cell epitope was encoded by a region located between nucleotides 1121 and 1288 of the COA-1 transcript. The longest open reading frame in the COA-1 transcript, which overlapped with the Socius gene product (20), was utilised as the basis for the synthesis of peptides that were used to identify the T cell epitope recognised by C111 T cells.

Peptides that were 20 or 21 amino acids in length and that overlapped by either 14 or 16 amino acids, were than synthesised and tested for their ability to sensitise target cells for recognition by C111 T cells. Since autologous normal B cells could not be efficiently expanded, allogeneic normal B cells expressing either DRβ1*0402 or DRβ1*1301 were used to carry out these assays.

The 1681 and 1847 CD40L stimulated normal B cell lines shared expression of HLA-DRβ1*0402 and HLA-DRβ1*1301 molecules, respectively, with the autologous tumour cell line. These cells were incubated with the panel of peptides and then tested for their ability to stimulate cytokine release from C111 T cells. The results showed that 1681 and 1847 CD40L B cells that were pulsed with either of the two overlapping peptides FSTFPPTLYQDDTLTLQAAG and TLYQDDTLTLQAAGLVPKAA stimulated significant cytokine release from C111 T cells.

These T cells thus recognise the peptide TLYQDDTLTLQAAG, which represents the overlapping region in these peptides. The L at position two, the T at the position 7 and L at position 10 in this sequence conform to an HLA binding motif that has been identified for the HLA-DRβ1*0402 class II allele (22). However, it was not possible to identify the potential anchor residues in this sequence that were involved in binding to the HLA- DRβ1*1301 allele. Nevertheless, these observations show that C111 T cells recognise a single peptide epitope in the context of either the HLA-DRβ1*0402 or 1301 class II gene products.

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Table 1 MHC Haplotype of cell lines

	A	B	C	DRβ1	DRβ3-5	DQ
1869	3,24	35,38	0401,1203	0402,1301	3*01,4*01	03,06
1870	24	35	04	1202	3*03	03
1872	02, 03	07, 4402	0501, 0702	0401,1501	4*01, 5*01	03, 06
1681	01,0201	08,44	N.D.	0301,0402	3*0101,4*01	0301,0402
1847	02	18, 44	05, 0701	0401,1301	3*01, 4*01	03, 06
1519	24,32	1401,4402	05,08	0701,1301	3*01,4*01	02, 06

Table 2 Specific recognition of colon cancer lines by CD4$^+$ clones from patient 1869.

Target cells	Antibody[a]	HLA-DRβ1	T cell[b]		
			C4	C49	C111
None	None		< 8[c]	< 8	< 8
1869 col + IFN-γ[d]	None	*0402, *1301	234	1213	536
	W6/32		212	1100	442
	L243		107	97	17
1869 col CIITA	None	*0402, *1301	536	5178	5005
	W6/32		527	4987	4249
	L243		47	254	305
1870 col + IFN-γ	None	*1202	< 8	< 8	< 8
	W6/32		< 8	< 8	< 8
	L243		< 8	< 7.8	< 8
1872 col + IFN-γ	None	*0401, *1501	< 8	< 8	< 8
	W6/32		< 8	< 8	< 8
	L243		< 8	< 8	< 8
SW 480 CIITA	None	*0103, *1301	879	968	963
	W6/32		780	902	996
	L243		571	129	127
Colo 205 CIITA	None	*0401, *1301	68	942	686
	W6/32		76	951	669
	L243		78	170	489
1869 EBV-B		*0402, *1301	52	126	322

a. Target cells were pre-incubated for 1 hour with either the anti-MHC class I mAb W6/32 or the anti-HLA DR mAb L243 before addition to T cells.

b. 2x10^4 T cells were incubated with 5x10^4 target cells in flat bottom 96-well plate in 250 μl of AIMV 2% HS. After 18 hrs. the supernatants IFN-γ secretion was evaluated by ELISA.

c. pg/ml of IFN-γ.

d. Where indicate, target cells were pre-incubated for 48 hrs with 500 IU of IFN-γ.

Table 3 CD4⁺ clones recognised colon cancer lines but not normal B or fibroblast cells sharing MHC class II molecules

A

Stimulator	Antibody[a]	HLA-DRβ1	T cell[b]				
			TIL		PBL		
			C5	C15	C4	C49	C111
None	None		<8 [c]	<8	<8	<8	<8
1869 col CIITA	None	*0402,	8695	1259	12328	12749	15269
	L243 [c]		279	162	511	524	790
1847 col + IFN-γ [d]	None	*0401,	2008	457	598	9758	11576
	L243		2055	327	585	790	2938
1872 col + IFN-γ	None	*0401,	72	<8	61	<8	66
	L243		75	<8	60	<8	41
1869 EBV-B	None	*0402,	79	116	122	232	209
1519 EBV-B	None	*0701,	112	24	99	106	220
1519 Fibroblast +	None	*0701,	<8	<8	<8	55	62
1869 CD40LB [e]	None	*0402,	<8	<8	<8	<8	45

B

Stimulator	Antibody [a]	T cell [b]
		C111
None		23
1869 col CIITA	-	15269
1869 col CIITA	HLA-DR [c]	790
1681 mel+ IFN-γ	-	10298
1681 mel+ IFN-γ	HLA-DR	253
1869 B cells	-	65
1681 B cells	-	22

a. Where indicated, target cells were pre-incubated for 1 hour with the anti-HLA DR mAb L243.
b. 2x10⁴ of the indicated T cells were incubated with 5x10⁴ target cells in flat bottom 96-well plate in 250 μl of AIMV 2% HS. After 18 hrs. the supernatants IFN-γ secretion was evaluated by ELISA.
c. pg/ml of IFN-γ.
d. Where indicated, target cells were pre-incubated for 48 hrs with 500 IU of IFN-γ.
e. B cells from the patient 1869 were in vitro cultured with CD40L (100 IU/ml) and IL-4 (100 IU/ml).

Table 4 Identification of the COA-1-derived epitopes recognised by the CD4+ clone C111.

Stimulator	HLA-DRβ1	No peptide
None		<8[a]
1869 col	0402, 1301	2186
1681 CD40LB	0301, 0402	<8
1847 CD40LB	0401, 1301	<8

Stimulator	HLA-DRβ1	Peptide[b]	Peptide Conc. (µg/ml)				
			100	50	25	12.5	6.25
1681 CD40LB	0301, 0402	FSTFPPTLYQDDTLTLQAAG	105	236	69	<7.8	<7.8
1681 CD40LB		TLYQDDTLTLQAAGLVPKAA	51	159	<7.8	<7.8	<7.8
1681 CD40LB		DDTLTLQAAGLVPKAALLLRA	11	16	<7.8	<7.8	<7.8
1681 CD40LB		LQAAGLVPKAALLLRARRAP	21	12	<7.8	<7.8	<7.8
1847 CD40LB	0401, 1301	ASAFEIFSTFPPTLYQDDTL	<7.8	<7.8	<7.8	<7.8	<7.8
1847 CD40LB		FSTFPPTLYQDDTLTLQAAG	226	397	296	79	<7.8
1847 CD40LB		TLYQDDTLTLQAAGLVPKAA	79	326	<7.8	<7.8	<7.8
1847 CD40LB		DDTLTLQAAGLVPKAALLLRA	22	33	<7.8	<7.8	<7.8
1847 CD40LB		LQAAGLVPKAALLLRARRAP	52	32	<7.8	<7.8	<7.8

a. The CD4+ T cell clone C111 was the added at 2×10^4 cells/well at the final volume of 250 µl/well of ISCOVE's plus 10 % HS and after 18 hrs. of incubation the supernatants were collected and the IFN-γ release was evaluated by ELISA.

b. Peptides of 20 or 21 amino acids overlapping by 15 amino acids were synthesised using the putative COA-1 protein, in the 1D8 region (1012-1318 bp). 4×10^5/ml of B cells sharing one of the DRβ1 molecules (*0402 or *1301) with the autologous tumour 1869, were incubated for three hrs. at 37°C and 5% CO_2 in the presence or not (-) of the peptides at the final volume of 100 µl/well in ISCOVE's plus 10% HS.

Copy provided by USPTO from the IFW Image Database on 02/18/2005

CLAIMS:

1.   A method for stimulating immunity against colorectal cancer, comprising stimulating the production of antibodies against the human equivalent of the Socius gene product, wherein the alanine residue at position 399 is substituted by a valine residue, wherein either or both of the coding sequence and the transcript thereof is as shown in Figure 5.

2.   A method for stimulating immunity against a substance as defined in claim 1, comprising the administration of an epitope located between amino acids 371 and 384, inclusive, of the COA-1 transcript in an immunity-stimulating form to the patient.

3.   A method according to claim 1 or 2, wherein immunity is stimulated by a peptide comprising the sequence TLYQDDTLTLQAAG.

4.   A method according to claim 3, wherein the peptide is an oligopeptide.

5.   A method according to any preceding claim, wherein immunity is stimulated in the patient in conjunction with PBMC's allogeneic or autologous for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

6.   A method according to claim 5, wherein the allele is selected from HLA DRβ1*0402 and HLA DRβ1*1301.

7.   A method according to any preceding claim, wherein the patient has PBMC's autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner, the method comprising administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any preceding claim, to the patient.

8.   Use of an immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, in the preparation of a medicament for the treatment or prophylaxis of colorectal cancer, wherein the patient has PBMC's autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

9. A method for stimulating immunity to colorectal cancer in a patient, said method comprising

i) isolating PBMC's or their progenitors from the patient and transforming said cells with at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner,

ii) introducing the transformed PBMC's back into the patient, and

iii) administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, to the patient.

10. A method according to claim 9, wherein the immunising portion of COA-1, or a precursor therefor, is administered with the transformed PBMC's.

11. A method for stimulating immunity to colorectal cancer in a patient, said method comprising administering a vaccine comprising the immunising portion of COA-1, or a precursor therefor, as defined in any of claims 1 to 4, to the patient, in conjunction with PBMC's obtained from a universal, or other suitable, donor for the patient, wherein said PBMC's are autologous or allogeneic for at least one sympathetic HLA-II allele capable of presenting the COA-1 epitope in an immunogenic manner.

12. A vaccine as defined in any preceding claim.

13. A nucleic acid encoding all or part of COA-1, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded.

14. A nucleic acid encoding COA-1 wherein the alanine residue at position 399 is substituted by a valine residue, provided that the immunising epitope provided between amino acids 371 and 384 of the wild type transcript is encoded.

15. The transcription product of a nucleic acid according to either of claims 13 and 14.

16. A host cell expressing COA-1, or a precursor therefor, provided that the immunising epitope is comprised in the transcript expressed thereby.

17. An antibody recognising COA-1 mutated at position 399.

18. A passive vaccine comprising an antibody according to claim 17.

19.   A diagnostic assay for colorectal cancer comprising an antibody according to claim 17.

36

ABSTRACT

COLORECTAL ANTIGEN

A point mutation in a novel, commonly expressed gene, designated as COA-1 herein, is diagnostic of colorectal cancer.

Fig.1A

Fig 1B

Fig 1C

Fig 2

Figure 3

Nucleotide sequence of COA-1 (the gCc triplet comprising C at nucleotide position 1280, encodes Alanine)

```
cgctgcggga  cggctagcgg  ccctgcgtgg  aggcgaggaa  tccgcatcta  tggagatgtc    60
cctgcatccc  atgactcgga  gctgatggcc  ttcatgacga  ggaagttgtg  ggacctggag   120
cagcaggtga  aggcccagac  tgatgagata  ctgtccaagg  atcagaagat  agcggcccta   180
gaggacctgg  tgcagaccct  ccggccacac  ccagccgagg  caaccctgca  gcggcaggag   240
gaactggaga  cgatgtgtgt  gcagctgcag  cggcaggtca  gggagatgga  gcggttcctc   300
agtgactatg  gcctgcagtg  ggtggggcgag  cccatggacc  aggaggactc  agagagcaag   360
acagtctcag  agcatggcga  gagggactgg  atgacagcca  agaagttctg  gaagccaggg   420
gactcattgg  cgcccccctga  ggtggacttt  gacaggctgc  tggccagcct  gcaggatctt   480
agtgagctgg  tggtagaggg  tgacacccaa  gtgacaccag  tgcccggcgg  ggcacggctg   540
cgtaccctcg  agcccatccc  gctgaagctc  taccggaatg  gcatcatgat  gttcgacggg   600
cccttccagc  ccttctacga  tccctccaca  cagcgctgcc  tccgagacat  attggatggc   660
ttctttccct  cagagctcca  gcgactgtac  cccaatgggg  tcccctttaa  ggtgagtgac   720
ttgcgcaatc  aggtctacct  ggaggatgga  ctggacccct  tcccaggcga  gggccgtgtg   780
gtgggcaggc  agcggatgca  caaggccttg  gacaggtgg   aggagcaccc  aggctccagg   840
atgactgctg  agaaatttct  gaacaggctc  cccaagtttt  gatccggcaa  ggcgaggtga   900
ttgacatccg  gggccccatc  agggacacct  tgcagaactg  ctgcccattg  cctgccgga   960
tccaggagat  tgtggtggag  acgcccacct  tggccgctga  gcgagagagg  agccaggagt  1020
cacccaacac  accggcaccc  ccgctctcca  tgctgcgcat  caagtctgag  aatggggaac  1080
aggccttcct  actgatgatg  cagcctgaca  acaccattgg  ggacgtgcga  gctctgctag  1140
cgcaggccag  ggtcatggat  gcctctgcct  ttgagatctt  cagcacattc  ccgcccaccc  1200
tctaccagga  cgatacactc  acgctgcagg  ctgcaggcct  tgtgcccaaa  gcagcactgc  1260
tgctgcgggc  acgccgagcc  ccgaagtcca  gcctgaaatt  cagtcctggt  ccctgtcccg  1320
gtcccggtcc  cggccccagt  cccggtcccg  gtcccggctc  cagtccctgt  cccggaccca  1380
gtcccagccc  ccaataaagc  acccaccccc  tc                                  1412
```

Protein Sequence of COA-1 (comprising Alanine at position 399)

```
MAFMTRKLWD  LEQQVKAQTD  EILSKDQKIA  ALEDLVQTLR  PHPAEATLQR  QEELETMCVQ    60
LQRQVREMER  FLSDYGLQWV  GEPMDQEDSE  SKTVSEHGER  DWMTAKKFWK  PGDSLAPPEV   120
DFDRLLASLQ  DLSELVVEGD  TQVTPVPGGA  RLRTLEPIPL  KLYRNGIMMF  DGPFQPFYDP   180
STQRCLRDIL  DGFFPSELQR  LYPNGVPFKV  SDLRNQVYLE  DGLDPFPGEG  RVVGRQRMHK   240
ALDRVEEHPG  SRMTAEKFLN  RLPKFVIRQG  EVIDIRGPIR  DTLQNCCPLP  ARIQEIVVET   300
PTLAAERERS  QESPNTPAPP  LSMLRIKSEN  GEQAFLLMMQ  PDNTIGDVRA  LLAQARVMDA   360
SAFEIFSTFP  PTLYQDDTLT  LQAAGLVPKA  ALLLRARRAP  KSSLKFSPGP  CPGPGPGPSP   420
GPGPGSSPCP  GPSPSPQ                                                      437
```

Alanine at
position 399

```
         10        20        30.       40        50        60
CGCTGCGGGACGGCTAGCGGCCCTGCGTGGAGGCGAGGAATCCGCATCTATGGAGATGTC

         70        80        90       100       110       120
CCTGCATCCCATGACTCGGAGCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAG
CAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAAGGGCTGATGGCCTTCATGACGA
GGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAAGG
GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGAC
TGATGAGATACTGTCCAAGGATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGACCCT
CCGGCCACACCCAGCCGAGGCAACCCTGCAGCGGCAGGAGGAACTGGAGACGATGTGTGT
GCAGCTGCAGCGGCAGGTCAGGGAGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAGTG
GGTGGGCGAGCCCATGGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGA
GAGGGACTGGATGACAGCCAAGAAGTTCTGGAAGCCAGGGGACTCATTGGCGCCCCCTGA
GGTGGACTTTGACAGGCTGCTGGCCAGCCTGCAGGATCTTAGTGAGCTGGTGGTAGAGGG
TGACACCCAAGTGACACCAGTGCCCGGCGGGGCACGGCTGCGTACCCTCGAGCCCATCCC
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGA
TCCCTCCACACAGCGCTGCCTCCGAGACATATTGGATGGCTTCTTTCCCTCAGAGCTCCA
GCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGACTTGCGCAATCAGGTCTACCT
GGAGGATGGACTGGACCCCTTCCCAGGCGAGGGCCGTGTGGTGGGCAGGCAGCGGATGCA
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTTCT
GAACAGGCTCCCCAAGTTTTGATCCGGCAAGGCGAGGTGATTGACATCCGGGGCCCCATCAGGGACACCTT
GCAGAACTGCTGCCCATTGCCTGCCCGG
ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGCTGAGCGAGAGAGGAGCCAGGAGTCACCCAACAC
ACCGGCACC
CCCGCTCTCCATGCTGCGCATCAAGTCTGAGAATGGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACA
ACACCATTG
GGGACGTGCGAGCTCTGCTAGCGCAGGCCAGGGTCATGGATGCCTCTGCCTTTGAGATCTTCAGCACATTC
CCGCCCACC
CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGGCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGC
ACGCCGAGC
CCCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCCTGTCCCGGTCCCGGTCCCGGCCCCAGTCCCGGTCCCG
GTCCCGGCT
CCAGTCCCTGTCCCGGACCCAGTCCCAGCCCCCAATAAAGCACCCACCCCCTC
SSPCPGPSPSPQ
```

```
         10        20        30        40        50        60
cgctgcgggacggctagcggccctgcgtggaggcgaggaatccgcatctatggagatgtc
         70        80        90       100       110       120
cctgcatcccatgactcggagctgatggccttcatgacgaggaagttgtgggacctggag
        130       140       150       160       170       180
cagcaggtgaaggcccagactgatgagatactgtccaaggatcagaagatagcggcccta
        190       200       210       220       230       240
gaggacctggtgcagaccctccggccacacccagccgaggcaaccctgcagcggcaggag
        250       260       270       280       290       300
gaactggagacgatgtgtgtgcagctgcagcggcaggtcagggagatggagcggttcctc
        310       320       330       340       350       360
agtgactatggcctgcagtgggtgggcgagcccatggaccaggaggactcagagagcaag
        370       380       390       400       410       420
acagtctcagagcatggcgagagggactggatgacagccaagaagttctggaagccaggg
        430       440       450       460       470       480
gactcattggcgccccctgaggtggactttgacaggctgctggccagcctgcaggatctt
        410       420       430       440       450       460
agtgagctggtggtagagggtgacacccaagtgacaccagtgcccggcggggcacggctg
        470       480       490       500       510       520
```

```
cgtaccctcgagcccatcccgctgaagctctaccggaatggcatcatgatgttcgacggg
        530       540       550       560       570       580
cccttccagcccttctacgatccctccacacagcgctgcctccgagacatattggatggc
        590       600       610       620       630       640
ttctttccctcagagctccagcgactgtaccccaatggggtccccttttaaggtgagtgac
        650       660       670       680       690       700
ttgcgcaatcaggtctacctggaggatggactggacccttcccaggcgagggccgtgtg
        710       720       730       740       750       760
gtgggcaggcagcggatgcacaaggccttggacagggtggaggagcacccaggctccagg
        770       780       790       800       810       820
atgactgctgagaaatttctgaacaggctcccccaagtttgatccggcaaggcgaggtga
        830       840       850       860       870       880
ttgacatccgggggccccatcagggacaccttgcagaactgctgcccattgcctgcccgga
        890       900       910       920       930       940
tccaggagattgtggtggagacgcccaccttggccgctgagcgagagaggagccaggagt
        950       960       970       980       990      1000
cacccaacacaccggcacccccgctctccatgctgcgcatcaagtctgagaatggggaac
       1010      1020      1030      1040      1050      1060
aggccttcctactgatgatgcagcctgacaacaccattggggacgtgcgagctctgctag
       1070      1080      1090      1100      1110      1120
cgcaggccagggtcatggatgcctctgcctttgagatcttcagcacattcccgcccaccc
       1130      1140      1150      1160      1170      1180
tctaccaggacgatacactcacgctgcaggctgcaggccttgtgcccaaagcagcactgc
       1190      1200      1210      1220      1230      1240
tgctgcgggcacgccgagcccccgaagtccagcctgaaattcagtcctggtccctgtcccg
       1250      1260      1270      1280      1290      1300
gtcccggtcccggccccagtcccggtcccggtcccggctccagtccctgtcccggaccca
       1370      1380      1390      1400      1410
gtcccagcccccaataaagcacccaccccctc
       1370      1380      1390      1400      1410
```

MAFMTRKLWDLEQQVKAQTDEILSK

DQKIAALEDLVQTLRPHPAEATLQRQE

ELETMCVQLQRQVREMERFLSDYGLQW

VGEPMDQEDSESKTVSEHGERDWMTA

KKFWKPGDSLAPPEVDFDRLLASLQDL

SELVVEGDTQVTPVPGGARLRTLEPIP

LKLYRNGIMMFDGPFQPFYDPSTQRC

LRDILDGFFPSELQRLYPNGVPFKVSD

LRNQVYLEDGLDPFPGEGRVVGRQRMH

KALDRVEEHPGSRMTAEKFLNRLPKF

VIRQGEVIDIRGPIRDTLQNCCPLPAR

IQEIVVETPTLAAERERSQESPNTPAP

PLSMLRIKSENGEQAFLLMMQPDNTI

GDVRALLAQARVMDASAFEI<u>FSTFPPT</u>

<u>LYQDDTLTLQAAG</u>LVPKAALLLRARRA
                                      t
V

                                                          COA-1a

                                                  ←_____

PKSSLKFSPGPCPGPGPGPSPGPGPG

Figure 4

IFN-γ (pg/ml)

```
          10         20         30         40         50         60         70         80
CGGCTGCGGGACGGCTAGCCCCTGCGCTGTGGAGGGCGAGGAATCCGCATCTATGGAGATGTCCCTGCATCCCATGACTCGGA

          90        100        110        120        130        140        150        160
GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGGTGAAGGCCCAGACTGATGAGATACTGTCCAAGG
 M  A  P  M  T  R  K  L  W  D  L  E  Q  Q  V  K  A  Q  T  D  E  I  L  S  K

         170        180        190        200        210        220        230        240
ATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGAACCCTCCGGCACACCCAGCCAGGCAACCTGCAGCCGGCAGGAG
 D  Q  K  I  A  A  L  E  D  L  V  Q  T  L  R  P  H  P  A  E  A  T  L  Q  R  Q  E

         250        260        270        280        290        300        310        320
GAACTGGAGACGATGTGTGTGCAGCTGCAGCTGCAGGTCAGGGAGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAGTG
 E  L  E  T  M  C  V  Q  L  Q  R  Q  V  R  E  M  E  R  F  L  S  D  Y  G  L  Q  W
                                                          ↑
                                                        COA-1a

         330        340        350        360        370        380        390        400
GGTGGGCGAGCCCATGGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGGACTGGATGACAGCCA
 V  G  B  P  M  D  Q  E  D  S  B  S  K  T  V  S  E  H  G  E  R  D  W  M  T  A

         410        420        430        440        450        460        470        480
AGAAGTTCTGGAAGCCAGGGGACTCATTGGCGCCCCTGAGGTGACTTTGACAGGCTGCTGGCCAGCCTGCAGGATCTT
 K  K  F  W  K  P  G  D  S  L  A  P  P  E  V  D  F  D  R  L  L  A  S  L  Q  D  L

         490        500        510        520        530        540        550        560
AGTGAGCTGGTGGTAGAGGGTGACACCCAAGTGACACCAGTGCCCGGCGGGCACGGCTGCGTACCCTCGAGCCCATCCC
 S  E  L  V  V  E  G  D  T  Q  V  T  P  V  P  G  G  A  R  L  R  T  L  E  P  I  P

         570        580        590        600        610        620        630        640
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCCTCCACACAGCCTGCC
 L  K  L  Y  R  N  G  I  M  M  F  D  G  P  F  F  Q  P  F  Y  D  P  S  T  Q  R  C

         650        660        670        680        690        700        710        720
TCCGAGACATATTGGATGGCTTCTTCTTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGAC
 L  R  D  I  L  D  G  F  F  P  S  E  L  Q  R  L  Y  P  N  G  V  P  F  K  V  S  D

         730        740        750        760        770        780        790        800
TTGCCAATCAGGTCTACCTGGAGGATGGACTGGACCCCTTCCCAGGCGAGGGCGAGGGCCGTGTGGTGGGCAGGCGGATGCA
 L  R  N  Q  V  Y  L  E  D  G  L  D  P  F  P  G  E  G  R  V  V  G  R  Q  R  M  H

         810        820        830        840        850        860        870        880
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTTCTGAACAGGCTCCCCAAGTTT
 K  A  L  D  R  V  E  E  H  P  G  S  R  M  T  A  E  K  F  L  N  R  L  P  K  F

         890        900        910        920        930        940        950        960
CAAGGGCCAAGGCGAGGTGATTGACATCCGGGGCCCCATCAGGACACCTTGCAGAACTGCTGCCCATTGCCTGCCCCGG
 V  I  R  Q  G  B  V  I  D  I  R  G  P  I  R  D  T  L  Q  N  C  C  P  L  P  A  R

         970        980        990       1000       1010       1020       1030       1040
```

FIG. 5

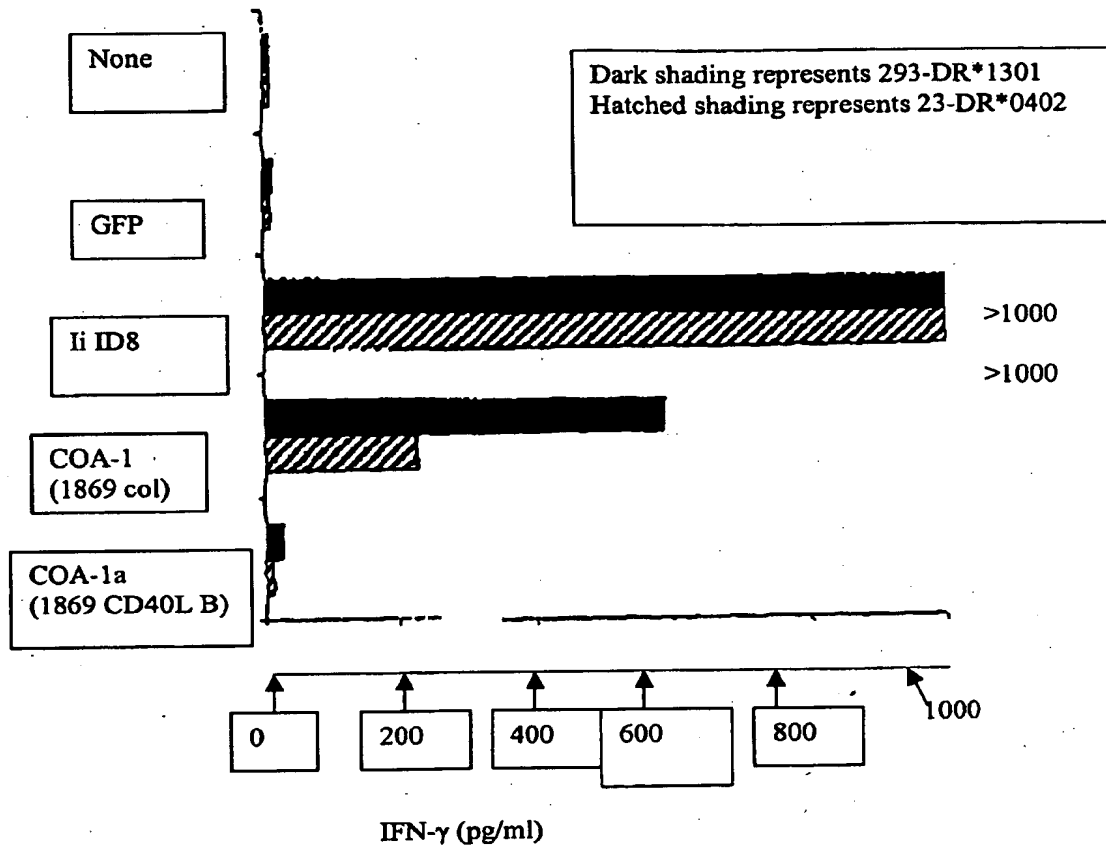Applicant(s):
COLORECTAL ANTIGEN

```
ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGGCTGAGCCGGCTGAGAGGAGGCCAGGAGTCACCGGCACC
 I  Q  E  I  V  V  E  T  P  T  L  A  A  E  R  E  R  S  Q  E  S  P  N  T  P  A  P
   1050      1060      1070      1080      1090      1100      1110      1120

CCCGGCTCTCGCATGCTGCGCATCAAGTCTGAGAATGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACAACACCATTG
 P  L  S  M  L  R  I  K  S  E  N  G  E  Q  A  F  L  L  M  M  Q  P  D  N  T  I
   1130      1140      1150      1160      1170      1180      1190      1200

GGGACGTGGCGAGCTCTGCTAGCGCAGGCCAGGGTCATGGATGCCTCTGCCTTTGAGATCTTCAGCACATTCCCGCCACC
 G  D  V  R  A  L  L  A  Q  A  R  V  M  D  A  S  A  F  E  I  F  S  T  F  P  P  T
   1210      1220      1230      1240      1250      1260      1270      1280

CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGGCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGCACGCCGAGC
 L  Y  Q  D  T  L  T  L  Q  A  A  G  L  V  P  K  A  A  L  L  L  R  A  R  R  A
                                                                            t
                                                                            v
                     COA-1a
   1290      1300      1310      1320      1330      1340      1350      1360

CCCGAAGTCCAGCCTGAAATTCAGTCCGGTCCCTGTCCCGGTCCCGGCCCCAGTCCCGGTCCCGGTCCCGGCT
 P  K  S  S  L  K  F  S  P  G  P  C  P  G  P  G  P  G  P  S  P  G  P  G  P  G

CCAGTCCCTGTCCCGGACCCAGTCCCAGCCCCCAATAAAGCACCCACCCCCCTC
 S  S  P  C  P  G  P  P  S  P  S  P  Q
   1370      1380      1390      1400      1410
```

Sequenza del gene COA-1 con traduzione della proteina

b) Sequenza della sequenza accorciata del gene COA-1 usata per le trasfezioni

```
     300       310       320       330       340       350       360       370       380       390       400
CCTCAGTGACTATGGCCTGCAGTGGGTGGGCGAGCCCATGGACCAGGAGCCTCAGAGAGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGACTGGATGACAGCCA
 L   S   D   Y   G   L   Q   W   V   G   E   P   M   D   Q   E   D   S   E   S   K   T   V   S   E   H   G   E   R   D   W   M   T   A

     410       420       430       440       450       460       470       480
AGAAGTTCTGGAAGCCAGGGGACTCATTGGCCGCCCCTGAGGTGGACTTTGACAGGCTGCTGGCCAGCCTGCAGGATCTT
 K   K   F   W   K   P   G   D   S   L   A   P   P   E   V   D   F   D   R   L   L   A   S   L   Q   D   L

     490       500       510       520       530       540       550       560
AGTGAGCTGGTGGTAGAGGGTGACACCCAAGTGACACCAGTGCCCGGCGGGCACGTGCGTACCCTGCGTACCCTCGAGCCCATCCC
 S   E   L   V   V   E   G   D   T   Q   V   T   P   V   P   G   G   A   R   L   R   T   L   E   P   I   P

     570       580       590       600       610       620       630       640
GCTGAAGCTCTACCGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCCTCCACACAGCGCTGCC
 L   K   L   Y   R   N   G   I   M   M   F   D   G   P   F   Q   P   F   Y   D   P   S   T   Q   R   C

     650       660       670       680       690       700       710       720
TCCGAGACATATTGGATGGCTTCTTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGAC
 L   R   D   I   L   D   G   F   F   P   S   E   L   Q   R   L   Y   P   N   G   V   P   F   K   V   S   D

     730       740       750       760       770       780       790       800
TTGCGCAATCAGGTCTACCTGGAGGATGGACTGGACCCCTTCCCAGGCGGAGGGCCGTGTGGTGGGCAGGCAGCGGGATGCA
 L   R   N   Q   V   Y   L   E   D   G   L   D   P   F   P   G   E   G   R   V   V   G   R   Q   R   M   H

     810       820       830       840       850       860       870       880
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGCAGAAATTTCTGAACAGGCTCCCAAGTTT
 K   A   L   D   R   V   E   E   H   P   G   S   R   M   T   A   E   K   F   L   N   R   L   P   K   F

     890       900       910       920       930       940       950       960
TGATCCGGCAAGGCGAGGTGATTGACATCCGGGGCCCCATCAGGGACACCTTGCAGAACTGCTGCCCATTGCCTGCCCCGG
 V   I   R   Q   G   E   V   I   D   I   R   G   P   I   R   D   T   L   Q   N   C   C   P   L   P   A   R

     970       980       990      1000      1010      1020      1030      1040
ATCCAGGAGATTGGTGGAGACGCCCACCTTGGCCGCGCTGAGCGAGGAGAGGAGCCAGGAGTCACCCAACACACCGGCACC
 I   Q   E   I   V   V   E   T   P   T   L   A   A   R   R   E   R   S   Q   E   S   P   N   T   P   A   P

    1050      1060      1070      1080      1090      1100      1110      1120
CCCGCTCTCCCATGCTGCGCATCAAGTCTGAGAAATGGGAACAGGCCTTCCTACTGATGATGCAGCCTGACAACACCATTG
 P   L   S   M   L   R   I   K   S   E   N   G   E   Q   A   F   L   L   M   M   Q   P   D   N   T   I

    1130      1140      1150      1160      1170      1180      1190      1200
GGGACGTGCGAGCTCTGCTAGCGCAGGCCAGGGTCATGGATGCCTCTGCCTTTGAGATCTTCAGCACATTCCGCCCACC
 G   D   V   R   A   L   L   A   Q   A   R   V   M   D   A   S   A   F   E   I   F   S   T   F   P   P   T

    1210      1220      1230      1240      1250      1260      1270      1280
CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGGCCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGCACGCCGAGC
 L   Y   Q   D   D   T   L   T   L   Q   A   A   G   L   V   P   K   A   A   L   L   R   A   R   R   A
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      1290      1300      1310      1320
CCCGAAGTCCAGCCTGAAATCAGTCCTGGTCCTGTCCCGGTCC
  P   K   S   L   K   F   S   P   G   P   C   P   G   P
```

v

La sequenza sottolineata corrisponde al peptide immunogenico che abbiamo isolato. La freccia indica la sostituzione di base (c →t)
e la conseguente sostituzione aminoacidica trovata nella sequenza isolata dalle cellule normali.

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CGCTGCGGGACGGCTAGCGGCCCCTGCGTGGAGGGCGAGGAATCCGCATCTATGGAGATGTCCCTGCATCCCATGACTCG
GA
GCTGATGGCCTTCATGACGAGGAAGTTGTGGGACCTGGAGCAGCAGTGAAGGCCCAGACTGATGAGATACTGTCCAA
GG
ATCAGAAGATAGCGGCCCTAGAGGACCTGGTGCAGACCCTCCGGCCACACCCAGCCGAGGCAACCCTGCAGCGGGCAGG
AG
GAACTGGAGACGATGTGTGTGCAGCTGCAGCGGCCAGGTCAGGGGAGATGGAGCGGTTCCTCAGTGACTATGGCCTGCAG
TG
GGTGGGGCGAGCCCATGACCAGGAGGACTCAGAGAGCAAGACAGTCTCAGAGCATGGCGAGAGGGACTGGATGACAG
CCA
AGAAGTTCTGGAAGCCAGGGGACTTCATTGGCGCCCCTGAGGTGGACTTTGACAGCTGCTGGCCAGCCTGCAGGATC
TT
AGTGAGCTGGTGGTAGAGGGTGACACCCAGTGCCCGGCGGGGCACGCTGCGTACCCTGAGCCCATC
CC
GCTGAAGCTCTACGGGAATGGCATCATGATGTTCGACGGGCCCTTCCAGCCCTTCTACGATCCCTCCACACAGGCTGC
C
TCCGAGACATATTGGATGGCTTCTTCCCTCAGAGCTCCAGCGACTGTACCCCAATGGGGTCCCCTTTAAGGTGAGTGA
C
TTGCGCCAATCAGGTCTCACCTGGAGGATGGACTGGACCCCTTCCCAGGCGAGGGGCCGTGTGGTGGGCAGGCAGCGGGATG
CA
CAAGGCCTTGGACAGGGTGGAGGAGCACCCAGGCTCCAGGATGACTGCTGAGAAATTCTGAACAGGCTCCCCAAGTT
TG
TGATCGGGCAAGGGCGAGGGTGATTGACATCCGGGGCCCCATCAGGAGACTGCTGCCATTGCCTGCCC
GG
ATCCAGGAGATTGTGGTGGAGACGCCCACCTTGGCCGCTGAGCGGAGCGAGAGGAGCCAGGAGTCACCCAACACACGGC
ACC
CCCGCTCTCCATGTGCGCATCAAGTCTGAGAATGGGGAACAGGCCTTCTACTGATGATGCAGCCTGACAACACCATT
G
GGGACGTGCGAGTCTGCTAGCGCAGGCGCCAGGGTCATGCGTCAGCGGCCTGCCTTGCCTTTGAGATCTTCAGCACATTCCCGCCCAC
C
CTCTACCAGGACGATACACTCACGCTGCAGGCTGCAGCCTTGTGCCCAAAGCAGCACTGCTGCTGCGGGGCACGCCGA
GC
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CCCGAAGTCCAGCCTGAAATTCAGTCCTGGTCCTGTCCCTGTCCCGGTCCCGGTCCCGGCCCCAGTCCCGGTCCCGGTCCCGGC
T
CCAGTCCCTGTCCCGGACCCAGTCCCAGCCCCCAATAAAGCACCCACCCCCTC